KALLIKREIN-INHIBITING "KUNITZ DOMAIN" PROTEINS AND ANALOGUES THEROF

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to novel classes of proteins and protein analogues which bind to and inhibit human plasma kallikrein.

Description of the Background Art

Kallikreins are serine proteases found in both tissues and plasma. Plasma kallikrein is involved in contact-activated (intrinsic pathway) coagulation, fibrinolysis, hypotension, and inflammation. (See BHOO92). These effects of kallikrein are mediated through the activities of three distinct physiological substrates: i) Factor XII (coagulation), ii) Pro-urokinase/plasminogen (fibrinolysis), and iii) Kininogens (hypotension and inflammation).

Kallikrein cleavage of kininogens results in the production of kinins, small highly potent bioactive peptides. The kinins act through cell surface receptors present on a variety of cell types. Intracellular heterotrimeric G-proteins link the kinin receptors to second messenger pathways including nitric oxide, adenyl cyclase, phospholipase A_2, and phospholipase C. Among the significant physiological activities of kinins are: (i) increased vascular permeability; (ii) vasodilation; (iii) bronchospasm; and (iv) pain induction. Thus, kinins mediate the life-threatening vascular shock and edema associated with bacteremia (sepsis) or trauma, the edema and airway hyperreactivity of asthma, and both inflammatory and neurogenic pain associated with tissue injury. The consequences of inappropriate plasma kallikrein activity and resultant kinin production are dramatically illustrated in patients with hereditary angioedema (HA). HA is due to a genetic deficiency of C1-inhibitor, the principal endogenous inhibitor of plasma kallikrein. Symptoms of HA include edema of the skin, subcutaneous tissues and gastrointestinal tract, and abdominal pain and vomiting. Nearly one-third of HA patients die by suffocation due to edema of the larynx and upper respiratory tract. Kallikrein is secreted as a zymogen (prekallikrein) that circulates as an inactive molecule until activated by a proteolytic event that frees the $+NH_3$-IVGGTNSS... sequence of kallikrein (SEQ ID NO. 1). Human Plasma Prekallikrein is found in Genebank entry

P03952.

Mature plasma Kallikrein contains 619 amino acids. Hydrolysis of the Arg_{371}-Ile_{372} peptide bond yields a two-chain proteinase joined by a disulfide bond. The amino-terminal light chain (248 residues) carries the catalytic site.

The main inhibitor of plasma kallikrein (pKA) *in vivo* is the C1 inhibitor; see SCHM87, pp.27-28. C1 is a serpin and forms an essentially irreversible complex with pKA. Although bovine pancreatic trypsin inhibitor (BPTI) was first said to be a strong pKA inhibitor with K_i = 320 pM (AUER88), BERN93 indicates that its K_i for pKA is 30 nM (*i.e.*, 30,000 pM). The G36S mutant had a K_i of over 500 nM. Thus, there is a need for a safe kallikrein inhibitor. The essential attributes of such an agent are:

i. Neutralization of relevant kallikrein enzyme(s);

ii. High affinity binding to target kallikreins to minimize dose;

iii. High specificity for kallikrein, to reduce side effects; and

iv. High degree of similarity to a human protein to minimize potential immunogenicity and organ/tissue toxicity.

The candidate target kallikreins to be inhibited are chymotrypin-homologous serine proteases.

Excessive Bleeding

Excessive bleeding can result from deficient coagulation activity, elevated fibrinolytic activity, or a combination of the two. In most diatheses one must controll the activity of plasmin. However, plasma kallikrein (pKA) is an activator of plasminogen and a potent, selective pKA inhibitor may avert plasminogen activation. The clinically beneficial effect of BPTI in reducing blood loss is thought to result from its inhibition of plasmin (K_D ~ 0.3 nM) or of plasma kallikrein (K_D ~ 100 nM) or both enzymes. It has been found, however, that BPTI is sufficiently antigenic that second uses require skin testing. Furthermore, the doses of BPTI required to control bleeding are quite high and the mechanism of action is not clear. Some say that BPTI acts on plasmin while others say that it acts by inhibiting plasma kallikrein. FRAE89 reports that doses of about 840 mg of BPTI to 80 open-heart surgery patients reduced blood loss by almost half and the mean amount transfused was decreased by 74%. Miles Inc. has recently introduced Trasylol in USA for reduction of bleeding in surgery (See Miles product brochure on Trasylol, which is hereby incorporated by reference.) LOHM93 suggests that plasmin inhibitors may be useful in controlling bleeding in surgery of the eye. SHER89 reports that BPTI may be useful in limiting

bleeding in colonic surgery.

A kallikrein inhibitor that is much more potent than BPTI and that is almost identical to a human protein domain offers similar therapeutic potential, allows dose to be reduced, and poses less potential for antigenicity.

With recombinant DNA techniques, one may obtain a novel protein by expression of a mutated gene of a parental protein. Several strategies are known for picking mutations to test. One, "protein surgery", involves the introduction of one or more predetermined mutations within the gene of choice. A single polypeptide of completely predetermined sequence is expressed, and its binding characteristics are evaluated.

At the other extreme is random mutagenesis by means of relatively nonspecific mutagens such as radiation and various chemical agents, see Lehtovaara, E.P. Appln. 285,123, or by expression of highly degenerate DNA. It is also possible to follow an intermediate strategy in which some residues are kept constant, others are randomly mutated, and still others are mutated in a predetermined manner. This is called "**variegation**". See Ladner, et al. USP 5,220,409.

DENN94a and DENN94b report selections of Kunitz domains based on APP-I for binding to the complex of Tissue Factor with Factor VII_a. They did not use LACI-K1 as parental and did not use pKA as a target. The highest affinity binder they obtained had K_D for their target of about 2 nM. Our first-round selectants for binding to pKA have affinity of about 0.3 nM, and our second round selectants are about at 0.1 nM (= 100 pM) or better.

Proteins taken from a particular species are assumed to be less likely to cause an immune response when injected into individuals of that species. Murine antibodies are highly antigenic in humans. "Chimeric" antibodies having human constant domains and murine variable domains are decidedly less antigenic. So called "humanized" antibodies have human constant domains and variable domains in which the CDRs are taken from murine antibodies while the framework of the variable domains are of human origin. "Humanized" antibodies are much less antigenic than are "chimeric" antibodies. In a "humanized" antibody, fifty to sixty residues of the protein are of non-human origin. The proteins of this invention comprise, in most cases, only about sixty amino acids and usually there are ten or fewer differences between the engineered protein and the parental protein. Although humans do develop antibodies even to human proteins, such as human insulin, such antibodies tend to bind weakly and the often do not prevent the injected protein from displaying its intended biological function. Using a protein from the species to be treated does

not guarantee that there will be no immune response. Nevertheless, picking a protein very close in sequence to a human protein greatly reduces the risk of strong immune response in humans.

Kunitz domains are highly stable and can be produced efficiently in yeast or other host organisms. At least ten human Kunitz domains have been reported. Although BPTI was thought at one time to be a potent pKA inhibitor, there are, actually, no human Kunitz domains that inhibits pKA very well. Thus, it is a goal of this invention to provide sequences of Kunitz domain that are both potent inhibitors of pKA and close in sequence to human Kunitz domains.

The use of site-specific mutagenesis, whether nonrandom or random, to obtain mutant binding proteins of improved activity, is known in the art, but does not guarantee that the mutant proteins will have the desired target specificity or affinity. Given the poor anti-kallikrein activity of BPTI, mutation of BPTI or other Kunitz domain proteins would not have been considered, prior to this invention, a preferred method of obtaining a strong binder, let alone inhibitor, of kallikrein.

SUMMARY OF THE INVENTION

This invention relates to novel BPTI-homologous Kunitz domains, especially LACI homologues, which inhibit one or more plasma (and/or tissue) kallikreins, and to the therapeutic and diagnostic use of these novel proteins. In particular, this invention relates to Kunitz domains derived from Kunitz domains of human origin and especially to the first Kunitz domain of LACI; Kunitz domains of human origin are likely to be non-immunogenic in humans. The proteins of this invention inhibit plasma kallikrein (and/or tissue kallikrein) with a K_D of no more than 20 nM, preferably, no more than 5 nM, more preferably, no more than about 300 pM, and most preferably, no more than about 100 pM.

A specific, high affinity inhibitor of plasma kallikrein (and, where needed, tissue kallikrein) will demonstrate significant therapeutic utility in all pathological conditions mediated by kallikrein, and especially those associated with kinins. The therapeutic approach of inhibiting the catalytic production of kinins is considered preferable to antagonism of kinin receptors, since in the absence of kallikrein inhibition, receptor antagonists must compete with continuous kinin generation. Significantly, genetic deficiency of plasma kallikrein is benign and thus, inhibition of plasma kallikrein is likely to be safe. We have recently discovered a lead pKA inhibitor, designated KKII/3#6. This inhibitor is a variant of a naturally occurring human plasma protein Kunitz domain

and demonstrates significantly greater kallikrein binding potency than Trasylol. KKII/3#6 has a K_i for kallikrein which is over 100 times that of both wild-type LACI and of BPTI, and is about 300 pM. In contrast, its K_i for plasmin is 10 μM. Proteins KK2/#11 and KK2/#13 are especially preferred pKA inhibitors and have K_i < 300 pM and probably less than 100 pM. A reversible inhibitor is believed to be of greater utility than an irreversible inhibitor such as the C1 inhibitor.

Transfer of the subsequences that confer pKA binding into other Kunitz domains, particularly human Kunitz domains is disclosed.

The preferred pKA inhibitors of the present invention fullfill one or more of the following desiderata:

1) the inhibitor inhibits plasma kallikrein with a K_i no more than 20 nM, preferably 5 nM or less, more preferably 300 pM or less, and most prefferably 100 pM or less,

2) the inhibitor comprise a Kunitz domain meeting the requirements shown in Table 14 with residues number by reference to BPTI,

3) the inhibitor has at the Kunitz domain positions 12-21 and 32-39 one of the amino-acid types listed for that position in Table 15, and

4) the inhibitor is substantially homologous to a reference sequence of essentially human origin selected from the group KKII/3#6, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, KK2con1, Human LACI-K2, Human LACI-K3, Human collagen α3 KuDom, Human TFPI-2 DOMAIN 1, Human TFPI-2 DOMAIN 2, Human TFPI-2 DOMAIN 3, HUMAN ITI-K1, Human ITI-K2, HUMAN PROTEASE NEXIN-II, Human APP-I, DKI-1.2.1, DKI-1.3.1, DKI-2.1, DKI-3.1.1, DKI-3.2.1, DKI-3.3.1, DKI-4.1.1, DKI-4.2.1, DKI-4.2.2, DKI-5.1, and DKI-6.1

NOMENCLATURE

Herein, affinities are stated as K_D ($K_D(A,B)=[A][B]/[A-B]$). A numerically smaller K_D reflects higher affinity. For the purposes of this invention, a "kallikrein inhibiting protein" is one that binds and inhibits a specified kallikrein with K_i of about 20 nM or less. "Inhibition" refers to blocking the catalytic activity of kallikrein and so is measurable *in vitro* in assays using chromogenic or fluorogenic substrates or in assays involving macromolecules.

Amino-acid residues are discussed in three ways: full name of the amino acid, standard

three-letter code, and standard single-letter code. The text uses full names and three-letter code where clarity requires.

A = Ala	G = Gly	M = Met	S = Ser
C = Cys	H = His	N = Asn	T = Thr
D = Asp	I = Ile	P = Pro	V = Val
E = Glu	K = Lys	Q = Gln	W = Trp
F = Phe	L = Leu	R = Arg	Y = Tyr

For the purposed of this invention, "**substantially homologous**" sequences are at least 51%, more preferably at least 80%, identical, over any specifiied regions. For this invention, "substantially homologous" includes exact identity. Sequences would still be "substantially homologous" if within one region of at least 20 amino acids they are sufficiently similar (51% or more) but outside the region of comparison they differed totally. An insertion of one amino acid in one sequence relative to the other counts as one mismatch. Most preferably, no more than six residues, other than at termini, are different. Preferably, the divergence in sequence, particularly in the specified regions, is in the form of "conservative modifications".

"**Conservative modifications**" are defined as

(a) conservative substitutions of amino acids as defined in Table 9; and

(b) single or multiple insertions or deletions of amino acids at termini, at domain boundaries, in loops, or in other segments of relatively high mobility.

Preferably, except at termini, no more than about six amino acids are inserted or deleted at any locus, and the modifications are outside regions known to contain important binding sites.

Kunitz Domains

Herein, "**Kunitz domain**" and "**KuDom**" are used interchangeably to mean a homologue of BPTI (not of the Kunitz soya-bean trypsin inhibitor). A KuDom is a domain of a protein having at least 51 amino acids (and up to about 61 amino acids) containing at least two, and preferably three, disulfides. Herein, the residues of **all Kunitz domains are numbered by reference to BPTI** (*i.e.* residues 1-58, amino-acid sequence in Table 2). Thus the first cysteine residue is residue 5 and the last cysteine is 55. An amino-acid sequence shall, for the purposed of this invention, be deemed a Kunitz domain if it can be aligned, with three or fewer mismatches, to the sequence shown in Table 14. An insertion or deletion of one residue shall count as one mismatch. In Table

14, "x" matches any amino acid and "X" matches the types listed for that position. Disulfides bonds link at least two of: 5 to 55, 14 to 38, and 30 to 51. The number of disulfides may be reduced by one, but none of the standard cysteines shall be left unpaired. Thus, if one cysteine is changed, then a compensating cysteine is added in a suitable location or the matching cysteine is also replaced by a non-cysteine (the latter being generally preferred). For example, *Drosophila funebris* male accessory gland protease inhibitor has no cysteine at position 5, but has a cysteine at position -1 (just before position 1); presumably this forms a disulfide to CYS_{55}. If Cys_{14} and Cys_{38} are replaced, the requirement of Gly_{12}, $(Gly\ or\ Ser)_{37}$, and Gly_{36} are dropped. From zero to many residues, including additional domains (including other KuDoms), can be attached to either end of a Kunitz domain.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Protease inhibitors, such as Kunitz domains, function by binding into the active site of the protease so that a peptide bond (the "scissile bond") is: 1) not cleaved, 2) cleaved very slowly, or 3) cleaved to no effect because the structure of the inhibitor prevents release or separation of the cleaved segments. In Kunitz domains, disulfide bonds act to hold the protein together even if exposed peptide bonds are cleaved. From the residue on the amino side of the scissile bond, and moving away from the bond, residues are conventionally called P1, P2, P3, *etc.* Residues that follow the scissile bond are called P1', P2', P3', *etc.* (SCHE67, SCHE68). It is generally accepted that each serine protease has sites (comprising several residues) S1, S2, *etc.* that receive the side groups and main-chain atoms of residues P1, P2, *etc.* of the substrate or inhibitor and sites S1', S2', *etc.* that receive the side groups and main-chain atoms of P1', P2', *etc.* of the substrate or inhibitor. It is the interactions between the S sites and the P side groups and main chain atoms that give the protease specificity with respect to substrates and the inhibitors specificity with respect to proteases. Because the fragment having the new amino terminus leaves the protease first, many worker designing small molecule protease inhibitors have concentrated on compounds that bind sites S1, S2, S3, *etc.*

LASK80 reviews protein protease inhibitors. Some inhibitors have several reactive sites on one polypeptide chain, and these domains usually have different sequences, specificities, and even topologies. It is known that substituting amino acids in the P_5 to P_5' region influences the specificity of an inhibitor. Previously, attention has been focused on the P1 residue and those very close to it because these can change the specificity from one enzyme class to another. LASK80

suggests that among KuDoms, inhibitors with P1=Lys or Arg inhibit trypsin, those with P1=Tyr, Phe, Trp, Leu and Met inhibit chymotrypsin, and those with P1=Ala or Ser are likely to inhibit elastase. Among the Kazal inhibitors, LASK80 continues, inhibitors with P1=Leu or Met are strong inhibitors of elastase, and in the Bowman-Kirk family elastase is inhibited with P1=Ala, but not with P1=Leu. Such limited changes do not provide inhibitors of truly high affinity (*i.e.* better than 1 to 10 nM).

KuDoms are defined above. The 3D structure (at high resolution) of BPTI (the archetypal Kunitz domain) is known. One of the X-ray structures is deposited in the Brookhaven Protein Data Bank as "6PTI"]. The 3D structure of some BPTI homologues (EIGE90, HYNE90) are known. At least seventy KuDom sequences are known. Known human homologues include three KuDoms of LACI (WUNT88, GIRA89, NOVO89), two KuDoms of Inter-α-Trypsin Inhibitor, APP-I (KIDO88), a KuDom from collagen, and three KuDoms of TFPI-2 (SPRE94).

LACI

Lipoprotein-associated coagulation inhibitor (LACI) is a human serum phosphoglycoprotein with a molecular weight of 39 kDa (amino-acid sequence in Table 1) containing three KuDoms. We refer hereinafter to the protein as LACI and to the Kunitz domains thereof as LACI-K1 (residues 50 to 107), LACI-K2 (residues 121 to 178), and LACI-K3 (213 to 270). The cDNA sequence of LACI is reported in WUNT88. GIRA89 reports mutational studies in which the P1 residues of each of the three KuDoms were altered. LACI-K1 inhibits Factor VIIa ($F.VII_a$) when $F.VII_a$ is complexed to tissue factor and LACI-K2 inhibits Factor X_a. It is not known whether LACI-K3 inhibits anything. Neither LACI nor any of the KuDoms of LACI is a potent plasma kallikrein inhibitor.

In one preferred embodiment of this invention, KuDoms are substantially homologous with LACI-K1, but differ in ways that confer strong plasma kallikrein inhibitory activity discussed below. Other KuDoms of this invention are homologous to other naturally-occurring KuDoms, particularly to other human KuDoms. For use in humans, the proteins of this invention are designed to be highly similar in sequence to one or another human KuDom to reduce the risk of causing an immune response.

Variegation of a protein is typically achieved by preparing a correspondingly variegated mixture of DNA (with variable codons encoding variable residues), cloning it into suitable vectors, and expressing the DNA in suitable host cells. For any given protein molecule of the

library, the choice of amino acid at each variable residue, subject to the above constraints, is random, the result of the happenstance of which DNA expressed that protein molecule.

FIRST LACI-K1 LIBRARY SCREENED FOR pKA BINDING

Applicants have screened a first large library of LACI-K1 domains (patern of variegation is shown in Table 21), with the results shown in Table 3. In Table 3, "Library Residues" are those permitted to occur, randomly, at that position, in the library, and "Preferred Residues" are those appearing at that position in at least one of the 10 variants identified as binding to human kallikrein.

At residues 13, 16, 17, 18, 31, and 32, the selections are very strong. At position 34, the selection for either SER or THR is quite strong. At position 39, the selection for GLY is strong. Position 19 seems to be rather tolerant.

It should be appreciated that Applicants have not sequenced all of the positive isolates in this or other the libraries herein disclosed, that some of the possible mutant proteins may not have been present in the library in detectable amounts, and that, at some positions, only some of the possible amino acids were intended to be included in the library.

SECOND LIBRARY OF LACI-K1 and SELECTION OF NEW KALLIKREIN INHIBITORS

Applicants prepared a second LACI-K1 library as shown in Table 750. This library utilized the observation of the first selection and allows variability at positions 10, 11, 13, 15, 16, 17, 18, 19, and 21. The residues at positions 34 and 39 were fixed at S_{34} and G_{39}. Selectants KK2/#1 through KK2/#13, as shown in Table 2 were obtained in the same manner as described in the Example section for the first screeneing. Applicants prepared the proteins KK2/#11 and KK2/#13 in *S. cerevisiae* in the Matα system described herein. Preliminary measurements indicate that these proteins are very potent pKA inhibitors with K_i less than 300 pM and probably less than 100 pM.

Using the selected sequences and the binding data of selected KuDoms, we can write a recipe for a high-affinity pKA-inhibiting KuDom that can be applied to other human KuDom parentals. First, the KuDom must meet the requirements in Table 14. The substitutions shown in Table 15 are likely to confer high-affinity pKA inhibitory activity on any KuDom. Thus a protein that contains a sequence that is a KuDom, as shown in Table 14, and that contains at each of the position 12-21 and 32-39 an amino-acid type shown in Table 15 for that position is likely

to be a potent inhibitor of human pKA. More preferably, the protein would have an amino-acid type shown in Table 15 for all of the positions listed in Table 15. To reduce the potential for immune response, one should use one or another human KuDom as parental protein to give the sequence outside the binding region.

It is likely that a protein that comprises an amino-acid sequence that is substantially homologous to one of KK2/#13, KK2/#11, or KKII/3#6 from residue 5 through residue 55 (as shown in Table 2) and is identical to one of KK2/#13, KK2/#11, or KKII/3#6 at positions 13-19, 31, 32, 34, and 39 will inhibit human pKA with a K_i of 5 nM or less. KK2/#13, KK2/#11, and KKII/3#6 differs from LACI-K1 at 10, 8 , and 7 positions respectively. It is not clear that these substitutions are equally important in fostering pKA binding and inhibition. From the known pKA inhibitors listed, one can prepare a series of molecules that are progressively reverted toward LACI-K1. It is expected that the molecules will show less affinity for pKA but also less potential for antigenicity. A person skilled in the art can pick a protein of sufficient potency and low immunogenicity from this collection. It is also possible that substitutions in one of the listed pKA inhibitors by amino acids that differ from LACI-K1 can reduce the immunogenicity without reducing the affinity for pKA to a degree that makes the protein unsuitable for use as a drug.

DESIGNED KuDom PKA Inhibitors

Hereinafter, "DKI" will mean a "Designed PKA Inhibitor" that are KuDoms that incorporate amino-acid sequence information from the SPI series of molecules, especially KK2/#13, KK2/#11, or KKII/3#6. Sequences of several DKIs and their parental proteins are given in Table 2. Hereinafter, the statement " the mutations $XnnY_1$, $XnnY_2$, ... may not be needed" means that each of the mutations might be separately found to be unnecessary. That is, the list is not to be taken as a block to be applied together, but as a list of things to be tested. Similarly, the lists of additional mutations are to be tested singly.

Protein DKI-1.2.1 is based on human **LACI-K2** and shown in Table 2. The mutations P11G, I13R, Y17A, I18H, T19P, Y21W, R32E, K34S, and L39G are likely to confer high affinity for pKA. Some of these substitutions may not be necessary; in particular, P11G and T19P may not be necessary. Other mutations that might improve the pKA affinity include E9A, D10E, G16A, Y21F, and L39E.

Protein DKI-1.3.1 (Table 2) is based on human **LACI-K3**. The mutations R11D, L13P, N17A, E18H, N19P, R31E, K34S, and S36G are intended to confer high affinity for pKA. Some

of these substitutions may not be necessary; in particular, N19P may not be necessary. Other changes that might improve K_D include D10E, F21W and G39E.

Protein DKI-2.1 (Table 2) is a based on the **human collagen α3 KuDom**. The mutations D16A, F17A, I18H, R32E, and W34S are likely to confer high affinity for pKA. Some of these substitutions may not be necessary; in particular, R32E may not be necessary. Other mutations that might improve the pKA affinity include K9A, D10E, D16G, K20R, R32T, W34V, and G39E.

DKI-3.1.1 (Table 2) is derived from **Human TFPI-2 domain 1**. The exchanges Y11G, L17A, L18H, R31E, and L34S are likely to confer high affinity for pKA. The mutation L34S may not be needed. Other mutations that might foster pKA binding include Y21W, Y21F, Q32E, L34T, L34I, and E39G.

DKI-3.2.1 (Table 2) is derived from **Human TFPI-2 domain 2**. This parental domain contains insertions after residue 9 (one residue) and 42 (two residues). The mutations E15R, G16A, S17A, T18H,E19P, K32T, and F34V are intended to confer affinity for pKA. If one needs a pKA inhibitor based on TFPI domain 2, a preferred route is to make a library of domains allowing the substitutions given and many others and then select binders.

DKI-3.3.1 (Table 2) is derived from **human TFPI-2, domain 3**. The substitutions L13H, S15R, and N17A are likely to confer high affinity for pKA. Other mutations that might foster pKA binding include D10E, T19Q, Y21W, T36G, and G39E.

DKI-4.1.1(Table 2) is from **human ITI-K1** by assertion of S10D, M15R, M17A, T18H, Q34S, and M39G. The mutations M39G and Q34V may not be necessary. Other mutations that should foster pKA binding include: G16A, M17N, S19Q, Y21W, and Y21F.

DKI-4.2.1(Table 2) is from **human ITI-K2** through the mutations V10D, R11D, F17A, I18H, V31E, L32E, P34S, and Q39E. The mutations V31E, L32E, and Q39E might not be necessary. Other mutation that should foster pKA binding include: V10E, Q19P, L20R, W21F, P34I, and Q39G. DKI-4.2.2 has eight mutations: V10D, R11D, F17A, I18H, L20R, V31E, L32E, and P34S.

DKI-5.1 is derived from human APP-I (also known as Protease Nexin-II) by mutations M17A, I18H, S19P, A31E, and P32E and is likely to be a potent pKA inhibitor. The mutations S19P, A31E, and P32E many not be needed. Other mutations that might foster pKA binding include T11D.

DKI-6.1 is derived from the **HKI B9 KuDom** (NORR93) by the five substitutions: K11D,

Q15R, T16A, M17A, M18H, T19P, and L32E. DKI-6.1 is likely to be a potent pKA inhibitor. The mutations L32E, and T19P might not be needed.

Although BPTI is not an especially good pKA inhibitor, it could be made into one. DKI-7.1 is derived from BPTI by the mutations Y10E, K15R, R17A, RI18H, I19P, Q31E, T32E, and R39E which is likely to increase the affinity for pKA. The mutations Y10E, K15R, I19P, Q31E, T32E, and R39E may not be needed; the really important mutations are R17A and RI18H.

MODIFICARION OF KUNITZ DOMAINS

KuDoms are quite small; if this should cause a pharmacological problem, such as excessively quick elimination from circulation, two or more such domains may be joined. A preferred linker is a sequence of one or more amino acids. A preferred linker is one found between repeated domains of a human protein, especially the linkers found in human BPTI homologues, one of which has two domains (BALD85, ALBR83a, ALBR83b) and another of which has three (WUNT88). Peptide linkers have the advantage that the entire protein may then be expressed by recombinant DNA techniques. It is also possible to use a nonpeptidyl linker, such as one of those commonly used to form immunogenic conjugates. An alternative means of increasing the serum residence of a BPTI-like KuDom is to link it to polyethyleneglycol, so called PEGylation (DAVI79).

WAYS TO IMPROVE SPECIFICITY OF, FOR EXAMPLE, KKII/3#7, KK2/#11, AND KK2/#13 FOR PLASMA KALLIKREIN:

Because we have made a large part of the surface of KKII/3#6, KK2/#11, and KK2/#13 complementary to the surface of pKA, R_{15} is not essential for specific binding to pKA. Many of the enzymes in the clotting and fibrinolytic pathways cut preferentially after Arg or Lys. Not having a basic residue at the P1 position may give rise to greater specificity. The variant KKII/3#7-K15A (shown in Table 27), having an ALA at P1, is likely to be a good pKA inhibitor and may have higher specificity for pKA relative to other proteases than doesKKII/3#7. The affinity of KKII/3#7-K15A for pKA is likely to be less than the affinity of KKII/3#7 for pKA, but the loss of affinity for other Arg/Lys-preferring enzymes is likely to be greater and, in many applications, specificity is more important than affinity. Other mutants that are likely to have good affinity and very high specificity include KK2/#13-R15A and KK2/#11-R15S. This approach could be applied to other high-affinity pKA inhibitors.

MODE OF PRODUCTION

The proteins of this invention may be produced by any conventional technique, including

(a) nonbiological synthesis by sequential coupling of component amino acids,

(b) production by recombinant DNA techniques in a suitable host cell, and

(c) removal of undesired sequences from LACI and coupling of synthetic replacement sequences

The proteins disclosed herein are preferably produced, recombinantly, in a suitable host, such as bacteria from the genera Bacillus, Escherichia, Salmonella, Erwinia, and yeasts from the genera Hansenula, Kluyveromyces, Pichia, Rhinosporidium, Saccharomyces, and Schizosaccharomyces, or cultured mammalian cells such as COS-1. The more preferred hosts are microorganisms of the species *Pichia pastoris*, *Bacillus subtilis*, *Bacillus brevis*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica*. Any promoter, regulatable or constitutive, which is functional in the host may be used to control gene expression.

Preferably the proteins are secreted. Most preferably, the proteins are obtained from conditioned medium. It is not required that the proteins described herein be secreted. Secretion is the preferred route because proteins are more likely to fold correctly, can be produced in conditioned medium with few contaminants, and are less likely to be toxic to host cells. Secretion is not required.

Unless there is a specific reason to include glycogroups, we prefer proteins designed to lack N-linked glycosylation sites to reduce potential for antigenicity of glycogroups and so that equivalent proteins can be expressed in a wide variety of organisms including: 1) *E. coli*, 2) *B. subtilis*, 3) *P. pastoris*, 4) *S. cerevisiae*, and 5) mammalian cells.

Several means exist for reducing the problem of host cells producing proteases that degrade the recombinant product; see, *inter alia* BANE90 and BANE91. VAND92 reports that overexpression of the *B. subtilis* signal peptidase in *E. coli*. leads to increased expression of a heterologous fusion protein. ANBA88 reports that addition of PMSF (a serine proteases inhibitor) to the culture medium improved the yield of a fusion protein.

Other factors that may affect production of these and other proteins disclosed herein include: 1) codon usage (optimizing codons for the host is preferred), 2) signal sequence, 3) amino-acid sequence at intended processing sites, presence and localization of processing

enzymes, deletion, mutation, or inhibition of various enzymes that might alter or degrade the engineered product and mutations that make the host more permissive in secretion (permissive secretion hosts are preferred).

Reference works on the general principles of recombinant DNA technology include Watson *et al.*, **Molecular Biology of the Gene**, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA (1987); Darnell *et al.*, **Molecular Cell Biology**, Scientific American Books, Inc., New York, N.Y. (1986); Lewin, **Genes II**, John Wiley & Sons, New York, N.Y. (1985); Old, *et al.*, **Principles of Gene Manipulation: An Introduction to Genetic Engineering**, 2d edition, University of California Press, Berkeley, CA (1981); Sambrook *et al.*, **Molecular Cloning: A Laboratory Manual**, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Ausubel *et al*, **Current Protocols in Molecular Biology**, Wiley Interscience, NY, (1987, 1992). These references are herein entirely incorporated by reference as are the references cited therein.

PREPARATION OF PEPTIDES

Chemical polypeptide synthesis is a rapidly evolving area in the art, and methods of solid phase polypeptide synthesis are well-described in the following references, hereby entirely incorporated by reference: (Merrifield, *J Amer Chem Soc* **85**:2149-2154 (1963); Merrifield, *Science* **232**:341-347 (1986); Wade *et al.*, *Biopolymers* **25**:S21-S37 (1986); Fields, *Int J Polypeptide Prot Res* **35**:161 (1990); MilliGen Report Nos. 2 and 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel *et al, supra*, and Sambrook *et al, supra.* Tan and Kaiser (*Biochemistry*, 1977, **16**:1531-41) synthesized BPTI and a homologue eighteen years ago.

As is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed. Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particles that can be filtered. Reactants are removed by washing the resin particles with appropriate solvents using an automated machine. Various methods, inclulding the "tBoc" method and the "Fmoc" method are well known in the art. See, *inter alia*, Atherton *et al.*, *J Chem Soc Perkin Trans* **1**:538-546 (1981) and Sheppard *et al.*, *Int J Polypeptide Prot Res* **20**:451-454 (1982).

ASSAYS FOR PLASMA KALLIKREIN BINDING AND INHIBITION

Any suitable method may be used to test the compounds of this invention. Scatchard (*Ann NY*

Acad Sci (1949) **51**:660-669) described a classical method of measuring and analyzing binding which is applicable to protein binding. This method requires relatively pure protein and the ability to distinguish bound protein from unbound.

A second appropriate method of measuring K_D is to measure the inhibitory activity against the enzyme. If the K_D to be measured is in the 1 nM to 1 μM range, this method requires chromogenic or fluorogenic substrates and tens of micrograms to milligrams of relatively pure inhibitor. For the proteins of this invention, having K_D in the range 5 nM to 50 pM, nanograms to micrograms of inhibitor suffice. When using this method, the competition between the inhibitor and the enzyme substrate can give a measured K_i that is higher than the true K_i. Measurement reported here are not so corrected because the correction would be very small and the any correction would reduce the K_i. Here, we use the measured K_i as a direct measure of K_D.

A third method of determining the affinity of a protein for a second material is to have the protein displayed on a genetic package, such as M13, and measure the ability of the protein to adhere to the immobilized "second material". This method is highly sensitive because the genetic packages can be amplified. We obtain at least semiquantitative values for the binding constants by use of a pH step gradient. Inhibitors of known affinity for the protease are used to establish standard profiles against which other phage-displayed inhibitors are judged. Any other suitable method of measuring protein binding may be used.

Preferably, the proteins of this invention have a K_D for pKA of at most about 5nM, more preferably at most about 300 pM, and most preferably 100 pM or less. Preferably, the binding is inhibitory so that K_i is the same as K_D. The K_i of KKII/3#6 is about 300 pM and the K_is of KK2/#11 and KK2/#13 are less than 300 pM and probably less than 100 pM.

PHARMACEUTICAL METHODS AND PREPARATIONS

The preferred subject of this invention is a mammal. The invention is particularly useful in the treatment of humans, but is suitable for veternary applications too.

Herein, "protection" includes "prevention", "suppression", and "treatment". "Prevention" involves administration of drug prior to the induction of disease. "Suppression" involves administration of drug prior to the clinical appearance of disease. "Treatment" involves administration of drug after the appearance of disease.

In human and veterinary medicine, it may not be possible to distinguish between "preventing" and "suppressing" since the inductive event(s) may be unknown or latent, or the patient is not ascertained until after the occurrence of the inductive event(s). We use the term

"prophylaxis" as distinct from "treatment" to encompass "preventing" and "suppressing". Herein, "protection" includes "prophylaxis". Protection need not by absolute to be useful.

Proteins of this invention may be administered, by any means, systemically or topically, to protect a subject against a disease or adverse condition. For example, administration of such a composition may be by any parenteral route, by bolus injection or by gradual perfusion. Alternatively, or concurrently, administration may be by the oral route. A suitable regimen comprises administration of an effective amount of the protein, administered as a single dose or as several doses over a period of hours, days, months, or years.

The suitable dosage of a protein of this invention may depend on the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the desired effect. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation by adjustment of the dose in ways known in the art.

For methods of preclinical and clinical testing of drugs, including proteins, see, e.g., Berkow *et al*, eds., **The Merck Manual**, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman *et al.*, eds., **Goodman and Gilman's The Pharmacological Basis of Therapeutics**, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); **Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics**, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, **Pharmacology**, Little, Brown and Co., Boston, (1985), which references and references cited there are hereby incorporated by reference.

In addition to a protein here disclosed, a pharmaceutical composition may contain pharmaceutically acceptable carriers, excipients, or auxiliaries. See, *e.g.*, Berker, *supra*, Goodman, *supra*, Avery, *supra* and Ebadi, *supra*.

IN VITRO DIAGNOSTIC METHODS AND REAGENTS

Proteins of this invention may be applied *in vitro* to any suitable sample that might contain plasma kallikrein to measure the pKA present. To do so, the assay must include a Signal Producing System (SPS) providing a detectable signal that depends on the amount of pKA present. The signal may be detected visually or instrumentally. Possible signals include production of colored, fluorescent, or luminescent products, alteration of the characteristics of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product.

The component of the SPS most intimately associated with the diagnostic reagent is called

the "label". A label may be, *e.g.*, a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, or an agglutinable particle. A radioactive isotope can be detected by use of, for example, a γ counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful are ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and, preferably, ^{125}I. It is also possible to label a compound with a fluorescent compound. When the fluorescently labeled compound is exposed to light of the proper wave length, its presence can be detected. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, and fluorescamine. Alternatively, fluorescence-emitting metals, such as ^{125}Eu or other lanthanide, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid or ethylenediamine-tetraacetic acid. The proteins also can be detectably labeled by coupling to a chemiluminescent compound, such as luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt, and oxalate ester. Likewise, a bioluminescent compound, such as luciferin, luciferase and aequorin, may be used to label the binding protein. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, are preferred.

There are two basic types of assays: heterogeneous and homogeneous. In heterogeneous assays, binding of the affinity molecule to analyte **does not** affect the label; thus, to determine the amount of analyte, bound label must be separated from free label. In homogeneous assays, the interaction **does** affect the activity of the label, and analyte can be measured without separation.

In general, a kallikrein-binding protein (KBP) may be used diagnostically in the same way that an anti-pKA antibody is used. Thus, depending on the assay format, it may be used to assay pKA, or, by competitive inhibition, other substances which bind pKA.

The sample will normally be a biological fluid, such as blood, urine, lymph, semen, milk, or cerebrospinal fluid, or a derivative thereof, or a biological tissue, *e.g.*, a tissue section or homogenate. The sample could be anything. If the sample is a biological fluid or tissue, it may be taken from a human or other mammal, vertebrate or animal, or from a plant. The preferred sample is blood, or a fraction or derivative thereof.

In one embodiment, the pKA-binding protein (KBP) is immobilized, and pKA in the sample is allowed to compete with a known quantity of a labeled or specifically labelable pKA analogue. The "pKA analogue" is a molecule capable of competing with pKA for binding to the KBP, which includes pKA itself. It may be labeled already, or it may be labeled subsequently by

specifically binding the label to a moiety differentiating the pKA analogue from pKA. The phases are separated, and the labeled pKA analogue in one phase is quantified.

In a "sandwich assay", both an insolubilized pKA-binding agent (KBA), and a labeled KBA are employed. The pKA analyte is captured by the insolubilized KBA and is tagged by the labeled KBA, forming a tertiary complex. The reagents may be added to the sample in any order. The KBAs may be the same or different, and only one KBA need be a KBP according to this invention (the other may be, *e.g.*, an antibody). The amount of labeled KBA in the tertiary complex is directly proportional to the amount of pKA in the sample.

The two embodiments described above are both heterogeneous assays. A homogeneous assay requires only that the label be affected by the binding of the KBP to pKA. The pKA analyte may act as its own label if a pKA inhibitor is used as a diagnostic reagent.

A label may be conjugated, directly or indirectly (*e.g.*, through a labeled anti-KBP antibody), covalently (*e.g.*, with SPDP) or noncovalently, to the pKA-binding protein, to produce a diagnostic reagent. Similarly, the pKA binding protein may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, and magnetite. The carrier can be soluble to some extent or insoluble for the purposes of this invention. The support material may have any structure so long as the coupled molecule is capable of binding pKA.

IN VIVO DIAGNOSTIC USES

A Kunitz domain that binds very tightly to pKA can be used for *in vivo* imaging. Diagnostic imaging of disease foci was considered one of the largest commercial opportunities for monoclonal antibodies, but this opportunity has not been achieved. Despite considerable effort, only two monoclonal antibody-based imaging agents have been approved. The disappointing results obtained with monoclonal antibodies is due in large measure to:

i) Inadequate affinity and/or specificity;

ii) Poor penetration to target sites;

iii) Slow clearance from nontarget sites;

iv) Immunogenicity (most are murine); and

v) High production cost and poor stability.

These limitations have led most in the diagnostic imaging field to begin to develop peptide-based imaging agents. While potentially solving the problems of poor penetration and slow clearance, peptide-based imaging agents are unlikely to possess adequate affinity, specificity and *in vivo*

stability to be useful in most applications.

Engineered proteins are uniquely suited to the requirements for an imaging agent. In particular the extraordinary affinity and specificity that is obtainable by engineering small, stable, human-origin protein domains having known *in vivo* clearance rates and mechanisms combine to provide earlier, more reliable results, less toxicity/side effects, lower production and storage cost, and greater convenience of label preparation. Indeed, it should be possible to achieve the goal of realtime imaging with engineered protein imaging agents. Thus, a Kallikrein-binding protein, e.g., KKII/3#6, KK2/#11, and KK2/#13 may be used for localizing sites of excessive pKA activity.

Radio-labelled binding protein may be administered to the human or animal subject. Administration is typically by injection, *e.g.*, intravenous or arterial or other means of administration in a quantity sufficient to permit subsequent dynamic and/or static imaging using suitable radio-detecting devices. The dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radio-imaging agents as guides.

Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The radio-labelled binding protein has accumulated. The amount of radio-labelled binding protein accumulated at a given point in time in relevant target organs can then be quantified.

A particularly suitable radio-detecting device is a scintillation camera, such as a γ camera. The detection device in the camera senses and records (and optional digitizes) the radioactive decay. Digitized information can be analyzed in any suitable way, many of which are known in the art. For example, a time-activity analysis can illustrate uptake through clearance of the radio-labelled binding protein by the target organs with time.

Various factors are taken into consideration in picking an appropriate radioisotope. The isotope is picked: to allow good quality resolution upon imaging, to be safe for diagnostic use in humans and animals, and, preferably, to have a short half-life so as to decrease the amount of radiation received by the body. The radioisotope used should preferably be pharmacologically inert, and the quantities administered should not have substantial physiological effect. The binding protein may be radio-labelled with different isotopes of iodine, for example ^{123}I, ^{125}I, or ^{131}I (see, for example, U.S. Patent 4,609,725). The amount of labeling must be suitably monitored.

In applications to human subjects, it may be desirable to use radioisotopes other than ^{125}I

for labelling to decrease the total dosimetry exposure of the body and to optimize the detectability of the labelled molecule. Considering ready clinical availability for use in humans, preferred radio-labels include: ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{90}Y, ^{111}In, ^{113m}In, ^{123}I, ^{186}Re, ^{188}Re or ^{211}At. Radio-labelled protein may be prepared by various methods. These include radio-halogenation by the chloramine-T or lactoperoxidase method and subsequent purification by high pressure liquid chromatography, for example, see Gutkowska *et al* in "Endocrinology and Metabolism Clinics of America: (1987) 16 (1):183. Other methods of radio-labelling can be used, such as IODOBEADS™.

A radio-labelled protein may be administered by any means that enables the active agent to reach the agent's site of action in a mammal. Because proteins are subject to digestion when administered orally, parenteral administration, *i.e.*, intravenous subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

High-affinity, high-specificity inhibitors are also useful for *in vitro* diagnostics of excess human pKA activity.

Other Uses

The kallikrein-binding proteins of this invention may also be used to purify kallikrein from a fluid, *e.g.*, blood. For this, the KBP is preferably immobilized on a support. Such supports, include those already mentioned as useful in preparing solid phase diagnostic reagents.

Proteins can be used as molecular weight markers for reference in the separation or purification of proteins. Proteins may need to be denatured to serve as molecular weight markers. A second general utility for proteins is the use of hydrolyzed protein as a nutrient source. Proteins may also be used to increase the viscosity of a solution.

The proteins of this invention may be used for any of the foregoing purposes, as well as for therapeutic and diagnostic purposes as discussed further earlier in this specification.

EXAMPLE 1: CONSTRUCTION OF FIRST LACI-K1 LIBRARY

A synthetic oligonucleotide duplex having *Nsi*I- and *Mlu*I-compatible ends was cloned into a parental vector (LACI:III) previously cleaved with the above two enzymes. The resultant ligated material was transfected by electroporation into XLIMR (F^-) *Escherichia coli* strain and plated on Amp plates to obtain phage-generating Ap^R colonies. The variegation scheme for Phase 1 focuses on the P1 region, and affected residues 13, 16, 17, 18 and 19. It allowed for 6.6×10^5 different DNA sequences (3.1×10^5 different protein sequences). The library obtained consisted of 1.4×10^6 independent cfu's which is approximately a two fold representation of the whole

library. The phage stock generated from this plating gave a total titer of 1.4×10^{13} pfu's in about 3.9 ml, with each independent clone being represented, on average, 1×10^{7} in total and 2.6×10^{6} times per ml of phage stock.

To allow for variegation of residues 31, 32, 34 and 39 (phase II), synthetic oligonucleotide duplexes with *Mlu*I- and *Bst*EII- compatible ends were cloned into previously cleaved R_f DNA derived from one of the following

i) the parental construction,

ii) the phase I library, or

iii) display phage selected from the first phase binding to a given target.

The variegation scheme for phase II allows for 4096 different DNA sequences (1600 different protein sequences) due to alterations at residues 31, 32, 34 and 39. The final phase II variegation is dependent upon the level of variegation remaining following the three rounds of binding and elution with a given target in phase I.

The combined possible variegation for both phases equals 2.7×10^{8} different DNA sequences or 5.0×10^{7} different protein sequences. When previously selected display phage are used as the origin of R_f DNA for the phase II variegation, the final level of variegation is probably in the range of 10^{5} to 10^{6}.

Example 2: Screening of LACI (K1) Library for Binding to Kallikrein

The overall scheme for selecting a LACI-K1 variant to bind to a given protease involves incubation of the phage-display library with the kallikrein-beads of interest in a buffered solution (PBS containing 1 mg/ml BSA) followed by washing away the unbound and poorly retained display-phage variant with PBS containing 0.1% Tween 20. Kallikrein beads were made by coupling human plasma Kallikrein (Calbiochem, San Diego, CA, # 420302) to agarose beads using Reactigel (6x) (Pierce, Rockford, Il, #202606). The more strongly bound display-phage are eluted with a low pH elution buffer, typically citrate buffer (pH 2.0) containing 1 mg/ml BSA, which is immediately neutralized with Tris buffer to pH 7.5. This process constitutes a single round of selection.

The neutralized eluted display-phage can be either used:

i) to inoculate an F^+ strain of *E. coli* to generate a new display-phage stock, to be used for subsequent rounds of selection (so-called conventional screening), or

ii) be used directly for another immediate round of selection with the protease beads (so-called quick screening).

Typically, three rounds of either method, or a combination of the two, are performed to give rise to the final selected display-phage from which a representative number are sequenced and analyzed for binding properties either as pools of display-phage or as individual clones.

Two phases of selection were performed, each consisting of three rounds of binding and elution. Phase I selection used the phase I library (variegated residues 13, 16, 17, 18, and 19) which went through three rounds of binding and elution against a target protease giving rise to a subpopulation of clones. The R_f DNA derived from this selected subpopulation was used to generate the Phase II library (addition of variegated residues 31, 32, 34 and 39). The 1.8×10^7 independent transformants were obtained for each of the phase II libraries. The phase II libraries underwent three further rounds of binding and elution with the same target protease giving rise to the final selectants.

Following two phases of selection against human plasma kallikrein-agarose beads a number (10) of the final selection display-phage were sequenced. The amino-acid sequences are shown in Table 2, entries KBPcon1 through KKII/3#C.

Table 23 shows that KkII/3(D) is a highly specific inhibitor of human Kallikrein. Phage that display the LACI-K1 derivative KkII/3(D) bind to Kallikrein beads at least 50-times more than it binds to other protease targets.

Preliminary measurements indicate that KKII/3#6 is a potent inhibitor of pKA with K_i probably less than 500 pM.

EXPRESSION, PURIFICATION AND KINETIC ANALYSIS.

The three isolates KKII/3#6, KK2/#11, and KK2/#13 were recloned into a yeast expression vector. The yeast expression vector is derived from pMFalpha8 (KURJ82 and MIYA85). The LACI variant genes were fused to part of the matα1 gene, generating a hybrid gene consisting of the matα1 promoter-signal peptide and leader sequence-fused to the LACI variant. The cloning site is shown in Table 24. Note that the correctly processed LACI-K1 variant protein should be as detailed in Table 2 with the addition of residues glu-ala-ala-glu to the N-terminal met (residue 1 in Table 2). Expression in *S. cerevisiae* gave acceptable yield typical of this system. Yeast-expressed LACI (kunitz domain 1), BPTI and LACI variants: KKII/3#6, KK2/#11, and KK2/#13 were purified by affinity chromatography using trypsin-agarose beads.

For larger-scale production, *Pichia pastoris* is a preferred host. The most preferred production sytem in *P. pastoris* is the alcohol oxidase system. Others have produced a number

of proteins in the yeast *Pichia pastoris*. For example, Vedvick *et al.* (VEDV91) and Wagner *et al.* (WAGN92) produced aprotinin from the alcohol oxidase promoter with induction by methanol as a secreted protein in the culture medium at ≈1 mg/ml. Gregg et al. (GREG93) have reviewed production of a number of proteins in P. pastoris. Table 1 of GREG93 shows proteins that have been produced in *P. pastoris* and the yields.

All references, including those to U.S. and foreign patents or patent applications, and to nonpatent disclosures, are hereby incorporated by reference in their entirety.

TABLE 1: Sequence of whole LACI:

```
    1   5           5           5           5           5
  1 MIYTMKKVHA LWASVCLLLN LAPAPLNAds eedeehtiit dtelpplklM
 51 HSFCAFKADD GPCKAIMKRF FFNIFTRQCE EFIYGGCEGN QNRFESLEEC
101 KKMCTRDnan riikttlqqe kpdfcfleed pgicrgyitr yfynnqtkqc
151 erfkyggclg nmnnfetlee cknicedgpn gfqvdnygtq lnavnnsltp
201 qstkvpslfe fh gpswcltp adrglcrane nrfyynsvig kcrpfkysgc
251 ggnennftsk qeclrackkg fiqriskggl iktkrkrkkq rvkiayeeif
301 vknm    (SEQ ID NO. 18)
```

The signal sequence (1-28) is uppercase and underscored
LACI-K1 is uppercase
LACI-K2 is underscored
LACI-K3 is bold

TABLE 2 is below.

Table 3: Summary of first selection of LACI-K1 domains for binding to pKA.

BPTI #	(Lac I)	Library Residues	Preferred Residues
13	P	LHPR	HP
16	A	AG	AG
17	I	FYLHINA SCPRTVD G	NSA
18	M	all	HL
19	K	LWQMKAG SPRTVE	QLP
31	E	EQ	E
32	E	EQ	EQ
34	I	all	STI
39	E	all	GEA

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444445555555551234567890123456789012345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
BPTI	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA	SEQ ID NO. 2
LACI-K1	mhsfcafkaddgpckaimkrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 3
KBPcon1	mhsfcafkaddgHckaNHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 4
KKII/3#1	mhsfcafkaddgHckASLPrfffniftrqcEEfIyggcEgnqnrfesleeckkmctrd	SEQ ID NO. 5
KKII/3#2	mhsfcafkaddgPckANHLrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 6
KKII/3#3	mhsfcafkaddgHckANHQrfffniftrqcEEfTyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 7
KKII/3#4	mhsfcafkaddgHckANHQrfffniftrqcEQfTyggcAgnqnrfesleeckkmctrd	SEQ ID NO. 8
KKII/3#5	mhsfcafkaddgHckASLPrfffniftrqcEEfIyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 9
KKII/3#6	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 10
KKII/3#7	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 11
KKII/3#8	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 12
KKII/3#9	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 13
KKII/3#10	mhsfcafkaddgHckGAHLrfffniftrqcEEfIyggcEgnqnrfesleeckkmctrd	SEQ ID NO. 14
KKI/3(a)	mhsfcafkaddgRckGAHLrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 15

PCT/US97/00299

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.		
Ident	Amino-acid sequence 1111111111222222222233333333334444444445555555551234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
KKI/3(b)	mhsfcafkaddgPckAIHLrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 16
KKII/3#C	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 17
KK2/#13	mhsfcafkaDGgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 19
KK2/#14	mhsfcafkaDGgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 20
KK2/#5	mhsfcafkaDDgPcRAAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 21
KK2/#11	mhsfcafkaDDgPcRAAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 22
KK2/#1	mhsfcafkaDVgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 23
KK2/#4	mhsfcafkaDVgRcRGAQPrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 24
KK2/#6	mhsfcafkaDDgScRAAHLrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 25
KK2/#10	mhsfcafkaEGgScRAAHQrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 26
KK2/#8	mhsfcafkaDDgPcRGAHLrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 27
KK2/#3	mhsfcafkaDDgHcRGALPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 28
KK2/#9	mhsfcafkaDSgNcRGNLPrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 29
KK2/#7	mhsfcafkaDSgRcRGNHQrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 30

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.		
Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
KK2/#12	mhsfcafkaDGgRcRAIQPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 31
KK2con1	mhsfcafkaDDgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 32
Human LACI-K2	KPDFCFLEEDPGICRGYITRYFYNNQTKQCERFKYGGCLGNMNNFETLEECKNICEDG	SEQ ID NO. 33
DKI-1.2.1	kpdfcfleedGgRcrgAHPrWfynnqtkqceEfSyggcGgnmnnfetleecknicedg	SEQ ID NO. 34
Human LACI-K3	GPSWCLTPADRGLCRANENRFYYNSVIGKCRPFKYSGCGGNENNFTSKQECLRACKKG	SEQ ID NO. 35
DKI-1.3.1	gpswcltpadDgPcraAHPrfyynsvigkcEpfSysgcggnennftskqeclrackkg	SEQ ID NO. 36
Human collagen α3 KuDom	ETDICKLPKDEGTCRDFILKWYYDPNTKSCARFWYGGCGGNENKFGSQKECEKVCAPV	SEQ ID NO. 37
DKI-2.1	etdicklpkdegtcrAAHlkwyydpntkscaEfSyggcggnenkfgsqkecekvcapv	SEQ ID NO. 38
TFPI-2 DOMAIN 1	NAEICLLPLDYGPCRALLLRYYYDRYTQSCRQFLYGGCEGNANNFYTWEACDDACWRI	SEQ ID NO. 39
DKI-3.1.1	naeicllpldGgpcraAHlryyydrytqscEqfSyggcegnannfytweacddacwri	SEQ ID NO. 40
tfpi-2 DOMAIN 2	VPKVCRLQVS- VDDQCEGSTEKYFFNLSSMTCEKFFSGGCHRNR- IENRFPDEATCMGFCAPK	SEQ ID NO. 41

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
DKI-3.2.1	vpkvcrlqvs- vddqcRAAHPkyffnlssmtceEffsggchrnr- ienrfpdeatcmgfcapk	SEQ ID NO. 42
TFPI-2 DOMAIN 3	IPSFCYSPKDEGLCSANVTRYYFNPRYRTCDAFTYTGCGGNDNNFVSREDCKRACAKA	SEQ ID NO. 43
DKI-3.3.1	ipsfcyspkdegHcRaAHQryyfnpryrtcdaftytgcggndnnfvsredckracaka	SEQ ID NO. 44
HUMAN ITI-K1	KEDSCQLGYSAGPCMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKECLQTCRTV	SEQ ID NO. 45
DKI-4.1.1	kedscqlgyDagpcRgAHPryfyngtsmacetfSyggcGgngnnfvtekeclqtcrtv	SEQ ID NO. 46
Human ITI-K2	TVAACNLPIVRGPCRAFIQLWAFDAVKGKCVLFPYGGCQGNGNKFYSEKECREYCGVP	SEQ ID NO. 47
DKI-4.2.1	tvaacnlpiDDgpcraAHqlwafdavkgkcEEfSyggcEgngnkfysekecreycgvp	SEQ ID NO. 48
DKI-4.2.2	tvaacnlpiDDgpcraAHqRwafdavkgkcEEfSyggcqgngnkfysekecreycgvp	SEQ ID NO. 49
HUMAN PROTEASE NEXIN-II	VREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSA	SEQ ID NO. 50
DKI-5.1	vrevcseqaetgpcraAHPrwyfdvtegkcEEfSyggcggnrnnfdteeycmavcgsa	SEQ ID NO. 51
HKI B9 domain	LPNVCAFPMEKGPCQTYMTRWFFNFETGECELFAYGGCGGNSNNFLRKEKCEKFCKFT	SEQ ID NO. 53
DKI-6.1	lpnvcafpmeDgpcRAAHPrwffnfetgeceEfayggcggnsnnflrkekcekfckft	SEQ ID NO. 54

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.		
Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
DKI-7.1	rpdfcleppEtgpcRaAHPryfynakaglcEEfvyggcGakrnnfksaedcmrtcgga	SEQ ID NO. 55

Table 3 is above.

TABLE 8: Binding Data for Selected Kallikrein-binding Display-Phage.

Display-Phage(a)	Fraction Bound(b)	Relative Binding(c)
LACI	4.2×10^{-6}	1.0
BPTI	2.5×10^{-5}	6.0
KKI/3(a)	3.2×10^{-3}	761
KKI/3(b)	2.2×10^{-3}	524
KKII/3#5	3.9×10^{-3}	928
KKII/3#6	8.7×10^{-3}	2071

(a) Clonal isolates of display-phage. LACI-K1 is the parental molecule, BPTI (bovine pancreatic trypsin inhibitor) is a control and KKII/3 (5 and 6) and KKI/3(a and b) were selected by binding to the target protease, kallikrein.

(b) The number of pfu's eluted after a binding experiment as a fraction of the input number (10^{10} pfu's).

(c) Fraction bound relative to the parental display-phage, LACI-K1.

Table 9: Conservative and Semiconservative substitutions

Initial AA type	Category	Conservative substitution	Semi-conservative substitution
A	Small non-polar or slightly polar	G, S, T	N, V, P, (C)
C	free SH	A, M, L, V, I	F, G
	disulfide	nothing	nothing
D	acidic, hydrophilic	E, N, S, T, Q	K, R, H, A
E	acidic, hydrophilic	D, Q, S, T, N	K, R, H, A
F	aromatic	W, Y, H, L, M	I, V, (C)
G	Gly-only conformation	nothing	nothing
	"normal" conformation	A, S, N, T	D, E, H, I, K, L, M, Q, R, V
H	amphoteric aromatic	Y, F, K, R	L, M, A, (C)
I	aliphatic, branched β carbon	V, L, M, A	F, Y, W, G (C)
K	basic	R, H	Q, N, S, T, D, E, A
L	aliphatic	M, I, V, A	F, Y, W, H, (C)
M	hydrophobic	L, I, V, A	Q, F, Y, W, (C), (R), (K), (E)
N	non-polar hydrophilic	S, T, (D), Q, A, G, (E)	K, R
P	inflexible	V, I	A, (C), (D), (E), F, H, (K), L, M, N, Q, (R), S, T, W, Y
Q	aliphatic plus amide	N, E, A, S, T, D	M, L, K, R
R	basic	K, Q, H	S, T, E, D, A,
S	hydrophilic	A, T, G, N	D, E, R, K

Table 9: Conservative and Semiconservative substitutions			
Initial AA type	Category	Conservative substitution	Semi-conservative substitution
T	hydrophilic	A, S, G, N, V	D, E, R, K, I
V	aliphatic, branched β carbon	I, L, M, A, T	P, (C)
W	aromatic	F, Y, H	L, M, I, V, (C)
Y	aromatic	F, W, H	L, M, I, V, (C)

Changing from A, F, H, I, L, M, P, V, W, or Y to C is semiconservative if the new cysteine remains as a free thiol.

Changing from M to E, R, K is semiconservative if the ionic tip of the new side group can reach the protein surface while the methylene groups make hydrophobic contacts.

Changing from P to one of K, R, E, or D is semiconservative if the side group is on or near the surface of the protein.

Table 14: Definition of a Kunitz Domain (SEQ ID NO. 52)
1 2 3 4 5 12345678901234567890123456789012345678901234567890123456789012345678 xxxxCxxxxxxGxCxxxxxxxXXXxxxxxxxCxxFxXXGCxXxxXxXxxxxxCxxxCxxx
Where: *X1, X2, X3, X4, X58, X57, and X56 may be absent,*
X21 = Phe, Tyr, Trp,
X22 = Tyr or Phe,
X23 = Tyr or Phe,
X35 = Tyr or Trp,
X36 = Gly or Ser,
X40 = Gly or Ala,
X43 = Asn or Gly, and
X45 = Phe or Tyr

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
10	**Asp**, Glu	Ala, Gly, Ser, Thr	Lys, Asn, (Arg, Cys, Phe, His, Ile, Leu, Met, Pro, Gln, Val, Trp, Tyr)
11	**Asp, Gly**, Ser, Val	Glu, Leu, Met, [Asn, Ile, Ala, Thr]	(Cys, Phe, His, Lys, Pro, Gln, Arg, Trp, Tyr)
12	**Gly**	(Other amino acids ONLY if C_{14}-C_{38} disulfide replaced by other amino acids.)	

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
13	**Arg, His**, Pro, Asn, Ser	[Thr, Ala, Gly, Lys, Gln]	Phe, Tyr, Cys, Leu, Ile, Val, Asp (Glu, Met, Trp)
14	**Cys**	(Other amino acids ONLY if C_{38} also changed.)	
15	**Arg**, Lys	[Ala, Ser, Gly, Met, Asn, Gln]	(Cys, Asp, Glu, Phe, His, Ile, Leu, Pro, Thr, Val, Trp, Tyr)
16	**Ala, Gly**	[Ser, Asp, Asn]	(Cys, Glu, Phe, His, Ile, Lys, Leu, Met, Pro, Gln, Arg, Thr, Val, Trp, Tyr)
17	**Ala, Asn**, Ser, Ile	[Gly, Val, Gln, Thr	Cys, Asp, Phe, His, Pro, Arg, Tyr, (Glu, Lys, Met, Trp)
18	**His**, Leu, Gln	[Ala,	Cys, Asp, Glu, Phe, Gly, Ile, Lys, Met, Asn, Pro, Arg, Ser, Thr, Val, Trp, Tyr
19	**Pro, Gln, Leu**	[Asn, Ile]	Ala, Glu, Gly, Met, Arg, Ser, Thr, Val, Trp, (Cys, Asp, Phe, His, Tyr)
20	**Arg**	Leu, Ala, Ser, Lys, Gln, Val	(Cys, Asp, Glu, Phe, Gly, His, Ile, Met, Asn, Pro, Thr, Trp, Tyr)

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
21	**Trp, Phe**	[Tyr, His, Ile]	Cys, Leu (Ala, Asp, Glu, Gly, Lys, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val)
31	**Glu**	[Asp, Gln, Asn, Ser, Ala, Val, Leu, Ile, Thr]	(Arg, Lys, Cys, Phe, Gly, His, Met, Pro, Trp, Tyr)
32	**Glu, Gln**	[Asp, Asn, Pro, Thr, Leu, Ser, Ala, Gly, Val]	(Cys, Phe, His, Ile, Lys, Met, Arg, Trp, Tyr)
33	**Phe**	[Tyr]	other 18 excluded.
34	**Ser, Thr, Ile**	[Val, Ala, Asn, Gly, Leu]	Cys, Asp, Glu, Phe, His, Lys, Met, Pro, Gln, Arg, Trp, Tyr
35	**Tyr**	[Trp, Phe]	(other 17)
36	**Gly**	Ser, Ala	(other 17)
37	**Gly**	(Other amino-acid types allowed only if C14-C38 replaced by other types.)	
38	**Cys**	(Other amino acids ONLY if C_{14} also changed.)	
39	**Gly, Glu, Ala**	[Ser, Asp]	Other 15.

Under "Preferred", most highly preferred type are **bold**

Under "Allowed" are types not actually tested, but judged to be acceptable. Types shown in square brackets were allowed and not selected, but are so similar to types that were selected

that the type is unlikely to abolish pKA binding. Such types are not preferred, but pKA-binding proteins could have such types.

Under "Unlikely to work", types shown outside parentheses have been tried and no isolates had that type; types in parentheses have not been tested, but are judged to be unsuitable from consideration of the types actually excluded.

TABLE 21: First Variegation of LACI-K1

	a	b	1	2	3	4	5	6	7	8	9	10
	A	E	M	H	S	F	C	A	F	K	A	D
	gcc	gag	atg	cat	tcc	ttc	tgc	gcc	ttc	aag	gct	gat
			NsiI									
							I	N				
							C	H				
						F	S	F	Y			
						Y	C	L	S	L	S	
						L	P	W	P	W	P	
						H	R	Q	R	Q	R	
						I	T	M	T	M	T	
						N	V	K	V	K	V	
		L	P			A	D	A	E	A	E	

	D	G	H	R	C	K	A	G	G	D	G	G	R
	11	12	13		14	15	16		17	18		19	20
	gat	ggt	cNt		tgt	aaa	gSt		NNt	NNS		NNg	cgt

	F	F	F	N	I	F	T	R	Q	C
	21	22	23	24	25	26	27	28	29	30
	ttc	ttc	ttc	aac	atc	ttc	acg	cgt	cag	tgc
							MluI			
				Q	E				N	H
				M	K				C	I
				F	S				F	Y
				Y	C				L	S
				L	P				W	P
				H	R				Q	R
				I	T				M	T
				N	V				K	V
				A	D				A	E

	E	Q	E	Q	F	G	W	Y	G	G	C	G	D	G	N	Q
	31		32		33	34		35	36	37	38	39		40	41	42
	Sag		Saa		ttc	NNS		tac	ggt	ggt	tgt	NNS		ggt	aac	cag
														BstEII		

Table 21, continued

```
                N   R   F   E   S   L   E   E
               43  44  45  46  47  48  49  50
              |aac|cgg|ttc|gaa|tct|cta|gag|gaa|
                        | BstBI |   | XbaI |
                 | AgeI |

      51  52  53  54  55  56  57  58  59  60
       C   K   K   M   C   T   R   D   G   A
     |tgt|aag|aag|atg|tgc|act|cgt|gac|ggc gcc
                                   | KasI  |
```

The segment from *Nsi*I to *Mlu*I gives 65,536 DNA sequences and 31,200 protein sequences. Second group of variegation gives 21,840 and 32,768 variants. This variegation can go in on a fragment having *Mlu*I and one of *Age*I, *Bst*BI, or *Xba*I ends. Because of the closeness between codon 42 and the 3' restriction site, one will make a self-priming oligonucleotide, fill in, and cut with *Mlu*I and, for example, *Bst*BI. Total variants are 2.726×10^9 and 8.59×10^9.

The DNA sequence has SEQ ID NO. 56.

The amino acid sequence has SEQ ID NO. 57.

TABLE 23: Specificity Results

Protein displayed on M13 gIIIp	Immobilized enzyme tested				
	Plasmin	Thrombin	pKA	Trypsin	Trypsin, 2 washes
LACI-K1	1	1	1	1	1
KkII/3(D)	3.4	1.5	196.	2.	1.4
BPTI	88.	1.1	1.7	0.3	.8

Numbers refer to relative binding of phage display clones compared to the parental phage display.

The KkII/3(D)(Kallikrein) clone retains the parental molecule's affinity for trypsin. KkII/3(D) was selected for binding to pKA.

TABLE 24: Mat α *S. cerevisiae* expression vectors:

Matα1 (Mfα8)

```
            K   R   P   R                                   SEQ ID NO. 58
  5'-...|AAA|AGG|CCT|CGA|G...-3'                            SEQ ID NO. 59
          | StuI  |
              | XhoI |
```

Matα2 (after introduction of a linker into *Stu*I-cut DNA)

```
                                                amino acids: SEQ ID NO. 60
    K   R   E   A   A   E   P   W   G   A   .   .   L   E
5'|AAA|AGG|GAA|GCG|GCC|GAG|CCA|TGG|GGC|GCC|TAA|TAG|CTC|GAG|3'
          | EagI |  | StyI  | KasI  |          | XhoI  |
                                                DNA: SEQ ID NO. 61
```

Matα-LACI-K1, amino acids: SEQ ID NO. 62, DNA: SEQ ID NO. 63

```
                a   b   c   d   1   2   3   4   5   6   7   8
    K   R   E   A   A   E   M   H   S   F   C   A   F   K
5'|AAA|AGG|GAA|GCG|GCC|GAG|atg|cat|tcc|ttc|tgc|gct|ttc|aaa|
          | EagI |  | NsiI  |

  9  10  11  12  13  14  15  16  17  18  19  20
  A   D   D   G   P   C   K   A   I   M   K   R
|gct|gat|gaC|ggT|ccG|tgt|aaa|gct|atc|atg|aaa|cgt|
        | RsrII |                | BspHI|

 21  22  23  24  25  26  27  28  29  30
  F   F   F   N   I   F   T   R   Q   C
|ttc|ttc|ttc|aac|att|ttc|acG|cgt|cag|tgc|
                    | MluI  |

 31  32  33  34  35  36  37  38  39  40  41  42
  E   E   F   I   Y   G   G   C   E   G   N   Q
|gag|gaA|ttC|att|tac|ggt|ggt|tgt|gaa|ggt|aac|cag|
    | EcoRI |                    | BstEII |

          43  44  45  46  47  48  49  50
           N   R   F   E   S   L   E   E
         |aac|cgG|ttc|gaa|tct|ctA|gag|gaa|
           |     | BstBI |  | XbaI |
           | AgeI |

  51  52  53  54  55  56  57  58  59  60
   C   K   K   M   C   T   R   D   G   A
|tgt|aag|aag|atg|tgc|act|cgt|gac|ggc|gcc|TAA|TAG|CTC|GAG|-3'
                            | KasI  |       | XhoI  |
```

We expect that Matα pre sequence is cleaved before GLU_a-ALA_b-

Table 27: High specificity plasma Kallikrein inhibitors

LACI-K1 (SEQ ID NO. 3)
MHSFCAFKADDGPCKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTRD
KKII/3#7 (SEQ ID NO. 11)
mhsfcafkaddg**H**ck**ANHQ**rfffniftrqc**EE**f**S**yggc**G**gnqnrfesleeckkmctrd
KKII/3#7-K15A (SEQ ID NO. 64)
mhsfcafkaddghc**A**anhqrfffniftrqceefsyggcggnqnrfesleeckkmctrd
KK2/#13-R15A (SEQ ID NO. 65)
mhsfcafkaDGgRc**A**GAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd
KK2/#11-R15S (SEQ ID NO. 66)
mhsfcafkaddgpc**S**aahprwffniftrqceefsyggcggnqnrfesleeckkmctrd

Table 49

	Residue Number											
	10	11	12	13	14	15	16	17	18	19	20	21
LACI-K1	D	D	G	P	C	K	A	I	M	K	R	F
Consensus of KKII/3 selectants	d	d	g	H	c	k	A	N	H	Q	r	f
KK Library #2	NK DE	NI AD ST GV	g	FS YC LP HR IT NV AD G	c	KR	AG	NI AD ST GV	QL HP R	QL HP R	r	FW CL
KK2/#13	-	G	-	-	-	-	-	-	-	-	-	-
KK2/#14	-	G	-	-	-	-	-	-	-	-	-	-
KK2/#5	-	-	-	P	-	-	A	-	-	-	-	-
KK2/#11	-	-	-	P	-	-	A	-	-	-	-	-
KK2/#1	-	V	-	-	-	-	-	-	-	-	-	-
KK2/#4	-	V	-	-	-	-	-	-	Q	-	-	F
KK2/#6	-	-	-	S	-	-	A	-	-	L	-	-
KK2/#10	E	G	-	S	-	-	A	-	-	Q	-	-
KK2/#8	-	-	-	P	-	-	-	-	-	L	-	F
KK2/#3	-	-	-	H	-	-	-	-	L	-	-	-
KK2/#9	-	S	-	N	-	-	-	N	L	-	-	F
KK2/#7	-	S	-	-	-	-	-	N	-	Q	-	F
KK2/#12	-	G	-	-	-	-	A	I	Q	-	-	-
Consensus #2	D	D	g	R	c	R	G	A	H	P	r	W

Table 750: DNA that embodies Library KKF.

```
                                                                  N|K
                    M    H    S    F    C    A    F    K    A   D|E
                    1    2    3    4    5    6    7    8    9   10
        5'-cctcct atg cat tcc ttc tgc gcc ttc aag gct RaS
                  | NsiI  |

              F|S
              Y|C
              L|H
              R|I
        I|V   V|T                   V|T  (M)  (K)
        T|A   A|G                   A|S  L|P  L|P
        S|G   D|N                   G|D  Q|R  R|H
        D|N   G    P    C   K|R A|G  I|N   H    Q    R
        11   12   13   14   15   16   17   18   19   20
        RNt  ggt  NNt  tgt  aRa  gSt  RNt  cNS  cNS  cgt

        W|L
        F|C   F    F    N    I    F    T    R   (SEQ ID NO. 69)
        21   22   23   24   25   26   27   28
        tKS  ttc  ttc  aac  atc  ttc  acg  cgt  tccctcc-3'  (SEQ ID NO. 67)
             3'-g  ttg  tag  aag  tgc  gca  agggagg-5'  (SEQ ID NO. 68)
                                | MluI  |
```

The *Rsr*II and *Bsp*HI sites found in the parental LACI-K1 display gene (Table 6) are not present in Library KKF.

There are 1,536,000 amino-acid sequences and 4,194,304 DNA sequences. Met_{18} and Lys_{19} are not allowed in Library KKF.

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CLAIMS

1. A kallikrein inhibiting protein which comprises a Kunitz Domain with residues numbered by reference to mature bovine pancreatic trypsin inhibitor, wherein, at each of the residues corresponding to the below identified residues of BPTI, one of the following allowed amino acids is found,

10	Asp, Glu
11	Asp, Gly, Ser, Val
13	His, Pro, Arg, Asn, Ser
15	Arg, Lys
16	Gly, Ala
17	Asn, Ser, Ala, Ile
18	His, Leu, Gln
19	Gln, Leu, Pro
21	Trp, Phe
31	Glu
32	Glu, Gln
34	Ser, Thr, Ile
39	Gly, Glu, Ala.

2. A plasma kallikrein inhibiting protein which comprises a sequence that is substantially homologuous to a reference sequence being selected from the group consisting of

KKII/3 # 1, KKII/3 # 2, KKII/3 # 3, KKII/3 # 4, KKII/3 # 5, KKII/3 # 6, KKII/3 # 7, KKII/3 # 8, KKII/3 # 9, KKII/3 # 10, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, and KK2con1 as defined in Table 2.

3. A method of preventing or treating a disorder attributable to excessive kallikrein activity which comprises administering, to a human or animal subject who would benefit therefrom, a kallikrein-inhibitory amount of the protein or analogue of claim 1 or claim 2.

4. A method of assaying for kallikrein which comprises providing the protein pr analogue of claim 1 or claim 2 in labeled or insolubilized form, and determining whether a complex of said protein and the kallikrein in a sample is formed.

5. A method of purifying kallikrein from a mixure which comprises providing the protein analogue of claim 1 in insolubilized form, and contacting the mixture with said insolubilized protein or analogue so that kallikrein in the mixture is bound.

PCT

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(57) Abstract

This invention relates to novel BPTI-homologous Kunitz domains, especially LACI homologues, which inhibit one or more plasma (and/or tissue) kallikreins, and to the therapeutic and diagnostic use of these novel proteins. In particular, this invention relates to Kunitz domains derived from Kunitz domains of human origin and especially to the first Kunitz domain of LACI.

KALLIKREIN-INHIBITING "KUNITZ DOMAIN" PROTEINS AND ANALOGUES THEROF

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to novel classes of proteins and protein analogues which bind to and inhibit human plasma kallikrein.

Description of the Background Art

Kallikreins are serine proteases found in both tissues and plasma. Plasma kallikrein is involved in contact-activated (intrinsic pathway) coagulation, fibrinolysis, hypotension, and inflammation. (See BHOO92). These effects of kallikrein are mediated through the activities of three distinct physiological substrates: i) Factor XII (coagulation), ii) Pro-urokinase/plasminogen (fibrinolysis), and iii) Kininogens (hypotension and inflammation).

Kallikrein cleavage of kininogens results in the production of kinins, small highly potent bioactive peptides. The kinins act through cell surface receptors present on a variety of cell types. Intracellular heterotrimeric G-proteins link the kinin receptors to second messenger pathways including nitric oxide, adenyl cyclase, phospholipase A_2, and phospholipase C. Among the significant physiological activities of kinins are: (i) increased vascular permeability; (ii) vasodilation; (iii) bronchospasm; and (iv) pain induction. Thus, kinins mediate the life-threatening vascular shock and edema associated with bacteremia (sepsis) or trauma, the edema and airway hyperreactivity of asthma, and both inflammatory and neurogenic pain associated with tissue injury. The consequences of inappropriate plasma kallikrein activity and resultant kinin production are dramatically illustrated in patients with hereditary angioedema (HA). HA is due to a genetic deficiency of C1-inhibitor, the principal endogenous inhibitor of plasma kallikrein. Symptoms of HA include edema of the skin, subcutaneous tissues and gastrointestinal tract, and abdominal pain and vomiting. Nearly one-third of HA patients die by suffocation due to edema of the larynx and upper respiratory tract. Kallikrein is secreted as a zymogen (prekallikrein) that circulates as an inactive molecule until activated by a proteolytic event that frees the $+NH_3$-IVGGTNSS... sequence of kallikrein (SEQ ID NO. 1). Human Plasma Prekallikrein is found in Genebank entry

P03952.

Mature plasma Kallikrein contains 619 amino acids. Hydrolysis of the Arg_{371}-Ile_{372} peptide bond yields a two-chain proteinase joined by a disulfide bond. The amino-terminal light chain (248 residues) carries the catalytic site.

The main inhibitor of plasma kallikrein (pKA) *in vivo* is the C1 inhibitor; see SCHM87, pp.27-28. C1 is a serpin and forms an essentially irreversible complex with pKA. Although bovine pancreatic trypsin inhibitor (BPTI) was first said to be a strong pKA inhibitor with K_i = 320 pM (AUER88), BERN93 indicates that its K_i for pKA is 30 nM (*i.e.*, 30,000 pM). The G36S mutant had a K_i of over 500 nM. Thus, there is a need for a safe kallikrein inhibitor. The essential attributes of such an agent are:

i. Neutralization of relevant kallikrein enzyme(s);

ii. High affinity binding to target kallikreins to minimize dose;

iii. High specificity for kallikrein, to reduce side effects; and

iv. High degree of similarity to a human protein to minimize potential immunogenicity and organ/tissue toxicity.

The candidate target kallikreins to be inhibited are chymotrypin-homologous serine proteases.

Excessive Bleeding

Excessive bleeding can result from deficient coagulation activity, elevated fibrinolytic activity, or a combination of the two. In most diatheses one must controll the activity of plasmin. However, plasma kallikrein (pKA) is an activator of plasminogen and a potent, selective pKA inhibitor may avert plasminogen activation. The clinically beneficial effect of BPTI in reducing blood loss is thought to result from its inhibition of plasmin (K_D ~ 0.3 nM) or of plasma kallikrein (K_D ~ 100 nM) or both enzymes. It has been found, however, that BPTI is sufficiently antigenic that second uses require skin testing. Furthermore, the doses of BPTI required to control bleeding are quite high and the mechanism of action is not clear. Some say that BPTI acts on plasmin while others say that it acts by inhibiting plasma kallikrein. FRAE89 reports that doses of about 840 mg of BPTI to 80 open-heart surgery patients reduced blood loss by almost half and the mean amount transfused was decreased by 74%. Miles Inc. has recently introduced Trasylol in USA for reduction of bleeding in surgery (See Miles product brochure on Trasylol, which is hereby incorporated by reference.) LOHM93 suggests that plasmin inhibitors may be useful in controlling bleeding in surgery of the eye. SHER89 reports that BPTI may be useful in limiting

bleeding in colonic surgery.

A kallikrein inhibitor that is much more potent than BPTI and that is almost identical to a human protein domain offers similar therapeutic potential, allows dose to be reduced, and poses less potential for antigenicity.

With recombinant DNA techniques, one may obtain a novel protein by expression of a mutated gene of a parental protein. Several strategies are known for picking mutations to test. One, "protein surgery", involves the introduction of one or more predetermined mutations within the gene of choice. A single polypeptide of completely predetermined sequence is expressed, and its binding characteristics are evaluated.

At the other extreme is random mutagenesis by means of relatively nonspecific mutagens such as radiation and various chemical agents, see Lehtovaara, E.P. Appln. 285,123, or by expression of highly degenerate DNA. It is also possible to follow an intermediate strategy in which some residues are kept constant, others are randomly mutated, and still others are mutated in a predetermined manner. This is called "**variegation**". See Ladner, et al. USP 5,220,409.

DENN94a and DENN94b report selections of Kunitz domains based on APP-I for binding to the complex of Tissue Factor with Factor VII_a. They did not use LACI-K1 as parental and did not use pKA as a target. The highest affinity binder they obtained had K_D for their target of about 2 nM. Our first-round selectants for binding to pKA have affinity of about 0.3 nM, and our second round selectants are about at 0.1 nM (= 100 pM) or better.

Proteins taken from a particular species are assumed to be less likely to cause an immune response when injected into individuals of that species. Murine antibodies are highly antigenic in humans. "Chimeric" antibodies having human constant domains and murine variable domains are decidedly less antigenic. So called "humanized" antibodies have human constant domains and variable domains in which the CDRs are taken from murine antibodies while the framework of the variable domains are of human origin. "Humanized" antibodies are much less antigenic than are "chimeric" antibodies. In a "humanized" antibody, fifty to sixty residues of the protein are of non-human origin. The proteins of this invention comprise, in most cases, only about sixty amino acids and usually there are ten or fewer differences between the engineered protein and the parental protein. Although humans do develop antibodies even to human proteins, such as human insulin, such antibodies tend to bind weakly and the often do not prevent the injected protein from displaying its intended biological function. Using a protein from the species to be treated does

not guarantee that there will be no immune response. Nevertheless, picking a protein very close in sequence to a human protein greatly reduces the risk of strong immune response in humans.

Kunitz domains are highly stable and can be produced efficiently in yeast or other host organisms. At least ten human Kunitz domains have been reported. Although BPTI was thought at one time to be a potent pKA inhibitor, there are, actually, no human Kunitz domains that inhibits pKA very well. Thus, it is a goal of this invention to provide sequences of Kunitz domain that are both potent inhibitors of pKA and close in sequence to human Kunitz domains.

The use of site-specific mutagenesis, whether nonrandom or random, to obtain mutant binding proteins of improved activity, is known in the art, but does not guarantee that the mutant proteins will have the desired target specificity or affinity. Given the poor anti-kallikrein activity of BPTI, mutation of BPTI or other Kunitz domain proteins would not have been considered, prior to this invention, a preferred method of obtaining a strong binder, let alone inhibitor, of kallikrein.

SUMMARY OF THE INVENTION

This invention relates to novel BPTI-homologous Kunitz domains, especially LACI homologues, which inhibit one or more plasma (and/or tissue) kallikreins, and to the therapeutic and diagnostic use of these novel proteins. In particular, this invention relates to Kunitz domains derived from Kunitz domains of human origin and especially to the first Kunitz domain of LACI; Kunitz domains of human origin are likely to be non-immunogenic in humans. The proteins of this invention inhibit plasma kallikrein (and/or tissue kallikrein) with a K_D of no more than 20 nM, preferably, no more than 5 nM, more preferably, no more than about 300 pM, and most preferably, no more than about 100 pM.

A specific, high affinity inhibitor of plasma kallikrein (and, where needed, tissue kallikrein) will demonstrate significant therapeutic utility in all pathological conditions mediated by kallikrein, and especially those associated with kinins. The therapeutic approach of inhibiting the catalytic production of kinins is considered preferable to antagonism of kinin receptors, since in the absence of kallikrein inhibition, receptor antagonists must compete with continuous kinin generation. Significantly, genetic deficiency of plasma kallikrein is benign and thus, inhibition of plasma kallikrein is likely to be safe. We have recently discovered a lead pKA inhibitor, designated KKII/3#6. This inhibitor is a variant of a naturally occurring human plasma protein Kunitz domain

and demonstrates significantly greater kallikrein binding potency than Trasylol. KKII/3#6 has a K_i for kallikrein which is over 100 times that of both wild-type LACI and of BPTI, and is about 300 pM. In contrast, its K_i for plasmin is 10 μM. Proteins KK2/#11 and KK2/#13 are especially preferred pKA inhibitors and have K_i < 300 pM and probably less than 100 pM. A reversible inhibitor is believed to be of greater utility than an irreversible inhibitor such as the C1 inhibitor.

Transfer of the subsequences that confer pKA binding into other Kunitz domains, particularly human Kunitz domains is disclosed.

The preferred pKA inhibitors of the present invention fullfill one or more of the following desiderata:

1) the inhibitor inhibits plasma kallikrein with a K_i no more than 20 nM, preferably 5 nM or less, more preferably 300 pM or less, and most prefferably 100 pM or less,

2) the inhibitor comprise a Kunitz domain meeting the requirements shown in Table 14 with residues number by reference to BPTI,

3) the inhibitor has at the Kunitz domain positions 12-21 and 32-39 one of the amino-acid types listed for that position in Table 15, and

4) the inhibitor is substantially homologous to a reference sequence of essentially human origin selected from the group KKII/3#6, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, KK2con1, Human LACI-K2, Human LACI-K3, Human collagen α3 KuDom, Human TFPI-2 DOMAIN 1, Human TFPI-2 DOMAIN 2, Human TFPI-2 DOMAIN 3, HUMAN ITI-K1, Human ITI-K2, HUMAN PROTEASE NEXIN-II, Human APP-I, DKI-1.2.1, DKI-1.3.1, DKI-2.1, DKI-3.1.1, DKI-3.2.1, DKI-3.3.1, DKI-4.1.1, DKI-4.2.1, DKI-4.2.2, DKI-5.1, and DKI-6.1

NOMENCLATURE

Herein, affinities are stated as K_D ($K_D(A,B)=[A][B]/[A-B]$). A numerically smaller K_D reflects higher affinity. For the purposes of this invention, a "kallikrein inhibiting protein" is one that binds and inhibits a specified kallikrein with K_i of about 20 nM or less. "Inhibition" refers to blocking the catalytic activity of kallikrein and so is measurable *in vitro* in assays using chromogenic or fluorogenic substrates or in assays involving macromolecules.

Amino-acid residues are discussed in three ways: full name of the amino acid, standard

three-letter code, and standard single-letter code. The text uses full names and three-letter code where clarity requires.

A = Ala	G = Gly	M = Met	S = Ser
C = Cys	H = His	N = Asn	T = Thr
D = Asp	I = Ile	P = Pro	V = Val
E = Glu	K = Lys	Q = Gln	W = Trp
F = Phe	L = Leu	R = Arg	Y = Tyr

For the purposed of this invention, "**substantially homologous**" sequences are at least 51%, more preferably at least 80%, identical, over any specifiied regions. For this invention, "substantially homologous" includes exact identity. Sequences would still be "substantially homologous" if within one region of at least 20 amino acids they are sufficiently similar (51% or more) but outside the region of comparison they differed totally. An insertion of one amino acid in one sequence relative to the other counts as one mismatch. Most preferably, no more than six residues, other than at termini, are different. Preferably, the divergence in sequence, particularly in the specified regions, is in the form of "conservative modifications".

"**Conservative modifications**" are defined as

(a) conservative substitutions of amino acids as defined in Table 9; and

(b) single or multiple insertions or deletions of amino acids at termini, at domain boundaries, in loops, or in other segments of relatively high mobility.

Preferably, except at termini, no more than about six amino acids are inserted or deleted at any locus, and the modifications are outside regions known to contain important binding sites.

Kunitz Domains

Herein, "**Kunitz domain**" and "**KuDom**" are used interchangeably to mean a homologue of BPTI (not of the Kunitz soya-bean trypsin inhibitor). A KuDom is a domain of a protein having at least 51 amino acids (and up to about 61 amino acids) containing at least two, and preferably three, disulfides. Herein, the residues of **all Kunitz domains are numbered by reference to BPTI** (*i.e.* residues 1-58, amino-acid sequence in Table 2). Thus the first cysteine residue is residue 5 and the last cysteine is 55. An amino-acid sequence shall, for the purposed of this invention, be deemed a Kunitz domain if it can be aligned, with three or fewer mismatches, to the sequence shown in Table 14. An insertion or deletion of one residue shall count as one mismatch. In Table

14, "x" matches any amino acid and "X" matches the types listed for that position. Disulfides bonds link at least two of: 5 to 55, 14 to 38, and 30 to 51. The number of disulfides may be reduced by one, but none of the standard cysteines shall be left unpaired. Thus, if one cysteine is changed, then a compensating cysteine is added in a suitable location or the matching cysteine is also replaced by a non-cysteine (the latter being generally preferred). For example, *Drosophila funebris* male accessory gland protease inhibitor has no cysteine at position 5, but has a cysteine at position -1 (just before position 1); presumably this forms a disulfide to CYS_{55}. If Cys_{14} and Cys_{38} are replaced, the requirement of Gly_{12}, $(Gly\ or\ Ser)_{37}$, and Gly_{36} are dropped. From zero to many residues, including additional domains (including other KuDoms), can be attached to either end of a Kunitz domain.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Protease inhibitors, such as Kunitz domains, function by binding into the active site of the protease so that a peptide bond (the "scissile bond") is: 1) not cleaved, 2) cleaved very slowly, or 3) cleaved to no effect because the structure of the inhibitor prevents release or separation of the cleaved segments. In Kunitz domains, disulfide bonds act to hold the protein together even if exposed peptide bonds are cleaved. From the residue on the amino side of the scissile bond, and moving away from the bond, residues are conventionally called P1, P2, P3, *etc.* Residues that follow the scissile bond are called P1', P2', P3', *etc.* (SCHE67, SCHE68). It is generally accepted that each serine protease has sites (comprising several residues) S1, S2, *etc.* that receive the side groups and main-chain atoms of residues P1, P2, *etc.* of the substrate or inhibitor and sites S1', S2', *etc.* that receive the side groups and main-chain atoms of P1', P2', *etc.* of the substrate or inhibitor. It is the interactions between the S sites and the P side groups and main chain atoms that give the protease specificity with respect to substrates and the inhibitors specificity with respect to proteases. Because the fragment having the new amino terminus leaves the protease first, many worker designing small molecule protease inhibitors have concentrated on compounds that bind sites S1, S2, S3, *etc.*

LASK80 reviews protein protease inhibitors. Some inhibitors have several reactive sites on one polypeptide chain, and these domains usually have different sequences, specificities, and even topologies. It is known that substituting amino acids in the P_5 to P_5' region influences the specificity of an inhibitor. Previously, attention has been focused on the P1 residue and those very close to it because these can change the specificity from one enzyme class to another. LASK80

suggests that among KuDoms, inhibitors with P1=Lys or Arg inhibit trypsin, those with P1=Tyr, Phe, Trp, Leu and Met inhibit chymotrypsin, and those with P1=Ala or Ser are likely to inhibit elastase. Among the Kazal inhibitors, LASK80 continues, inhibitors with P1=Leu or Met are strong inhibitors of elastase, and in the Bowman-Kirk family elastase is inhibited with P1=Ala, but not with P1=Leu. Such limited changes do not provide inhibitors of truly high affinity (*i.e.* better than 1 to 10 nM).

KuDoms are defined above. The 3D structure (at high resolution) of BPTI (the archetypal Kunitz domain) is known. One of the X-ray structures is deposited in the Brookhaven Protein Data Bank as "6PTI"]. The 3D structure of some BPTI homologues (EIGE90, HYNE90) are known. At least seventy KuDom sequences are known. Known human homologues include three KuDoms of LACI (WUNT88, GIRA89, NOVO89), two KuDoms of Inter-α-Trypsin Inhibitor, APP-I (KIDO88), a KuDom from collagen, and three KuDoms of TFPI-2 (SPRE94).

LACI

Lipoprotein-associated coagulation inhibitor (LACI) is a human serum phosphoglycoprotein with a molecular weight of 39 kDa (amino-acid sequence in Table 1) containing three KuDoms. We refer hereinafter to the protein as LACI and to the Kunitz domains thereof as LACI-K1 (residues 50 to 107), LACI-K2 (residues 121 to 178), and LACI-K3 (213 to 270). The cDNA sequence of LACI is reported in WUNT88. GIRA89 reports mutational studies in which the P1 residues of each of the three KuDoms were altered. LACI-K1 inhibits Factor VIIa ($F.VII_a$) when $F.VII_a$ is complexed to tissue factor and LACI-K2 inhibits Factor X_a. It is not known whether LACI-K3 inhibits anything. Neither LACI nor any of the KuDoms of LACI is a potent plasma kallikrein inhibitor.

In one preferred embodiment of this invention, KuDoms are substantially homologous with LACI-K1, but differ in ways that confer strong plasma kallikrein inhibitory activity discussed below. Other KuDoms of this invention are homologous to other naturally-occurring KuDoms, particularly to other human KuDoms. For use in humans, the proteins of this invention are designed to be highly similar in sequence to one or another human KuDom to reduce the risk of causing an immune response.

Variegation of a protein is typically achieved by preparing a correspondingly variegated mixture of DNA (with variable codons encoding variable residues), cloning it into suitable vectors, and expressing the DNA in suitable host cells. For any given protein molecule of the

library, the choice of amino acid at each variable residue, subject to the above constraints, is random, the result of the happenstance of which DNA expressed that protein molecule.

FIRST LACI-K1 LIBRARY SCREENED FOR pKA BINDING

Applicants have screened a first large library of LACI-K1 domains (patern of variegation is shown in Table 21), with the results shown in Table 3. In Table 3, "Library Residues" are those permitted to occur, randomly, at that position, in the library, and "Preferred Residues" are those appearing at that position in at least one of the 10 variants identified as binding to human kallikrein.

At residues 13, 16, 17, 18, 31, and 32, the selections are very strong. At position 34, the selection for either SER or THR is quite strong. At position 39, the selection for GLY is strong. Position 19 seems to be rather tolerant.

It should be appreciated that Applicants have not sequenced all of the positive isolates in this or other the libraries herein disclosed, that some of the possible mutant proteins may not have been present in the library in detectable amounts, and that, at some positions, only some of the possible amino acids were intended to be included in the library.

SECOND LIBRARY OF LACI-K1 and SELECTION OF NEW KALLIKREIN INHIBITORS

Applicants prepared a second LACI-K1 library as shown in Table 750. This library utilized the observation of the first selection and allows variability at positions 10, 11, 13, 15, 16, 17, 18, 19, and 21. The residues at positions 34 and 39 were fixed at S_{34} and G_{39}. Selectants KK2/#1 through KK2/#13, as shown in Table 2 were obtained in the same manner as described in the Example section for the first screeneing. Applicants prepared the proteins KK2/#11 and KK2/#13 in *S. cerevisiae* in the Matα system described herein. Preliminary measurements indicate that these proteins are very potent pKA inhibitors with K_i less than 300 pM and probably less than 100 pM.

Using the selected sequences and the binding data of selected KuDoms, we can write a recipe for a high-affinity pKA-inhibiting KuDom that can be applied to other human KuDom parentals. First, the KuDom must meet the requirements in Table 14. The substitutions shown in Table 15 are likely to confer high-affinity pKA inhibitory activity on any KuDom. Thus a protein that contains a sequence that is a KuDom, as shown in Table 14, and that contains at each of the position 12-21 and 32-39 an amino-acid type shown in Table 15 for that position is likely

to be a potent inhibitor of human pKA. More preferably, the protein would have an amino-acid type shown in Table 15 for all of the positions listed in Table 15. To reduce the potential for immune response, one should use one or another human KuDom as parental protein to give the sequence outside the binding region.

It is likely that a protein that comprises an amino-acid sequence that is substantially homologous to one of KK2/#13, KK2/#11, or KKII/3#6 from residue 5 through residue 55 (as shown in Table 2) and is identical to one of KK2/#13, KK2/#11, or KKII/3#6 at positions 13-19, 31, 32, 34, and 39 will inhibit human pKA with a K_i of 5 nM or less. KK2/#13, KK2/#11, and KKII/3#6 differs from LACI-K1 at 10, 8 , and 7 positions respectively. It is not clear that these substitutions are equally important in fostering pKA binding and inhibition. From the known pKA inhibitors listed, one can prepare a series of molecules that are progressively reverted toward LACI-K1. It is expected that the molecules will show less affinity for pKA but also less potential for antigenicity. A person skilled in the art can pick a protein of sufficient potency and low immunogenicity from this collection. It is also possible that substitutions in one of the listed pKA inhibitors by amino acids that differ from LACI-K1 can reduce the immunogenicity without reducing the affinity for pKA to a degree that makes the protein unsuitable for use as a drug.

DESIGNED KuDom PKA Inhibitors

Hereinafter, "DKI" will mean a "Designed PKA Inhibitor" that are KuDoms that incorporate amino-acid sequence information from the SPI series of molecules, especially KK2/#13, KK2/#11, or KKII/3#6. Sequences of several DKIs and their parental proteins are given in Table 2. Hereinafter, the statement " the mutations $XnnY_1$, $XnnY_2$, ... may not be needed" means that each of the mutations might be separately found to be unnecessary. That is, the list is not to be taken as a block to be applied together, but as a list of things to be tested. Similarly, the lists of additional mutations are to be tested singly.

Protein DKI-1.2.1 is based on human **LACI-K2** and shown in Table 2. The mutations P11G, I13R, Y17A, I18H, T19P, Y21W, R32E, K34S, and L39G are likely to confer high affinity for pKA. Some of these substitutions may not be necessary; in particular, P11G and T19P may not be necessary. Other mutations that might improve the pKA affinity include E9A, D10E, G16A, Y21F, and L39E.

Protein DKI-1.3.1 (Table 2) is based on human **LACI-K3**. The mutations R11D, L13P, N17A, E18H, N19P, R31E, K34S, and S36G are intended to confer high affinity for pKA. Some

of these substitutions may not be necessary; in particular, N19P may not be necessary. Other changes that might improve K_D include D10E, F21W and G39E.

Protein DKI-2.1 (Table 2) is a based on the **human collagen α3 KuDom**. The mutations D16A, F17A, I18H, R32E, and W34S are likely to confer high affinity for pKA. Some of these substitutions may not be necessary; in particular, R32E may not be necessary. Other mutations that might improve the pKA affinity include K9A, D10E, D16G, K20R, R32T, W34V, and G39E.

DKI-3.1.1 (Table 2) is derived from **Human TFPI-2 domain 1**. The exchanges Y11G, L17A, L18H, R31E, and L34S are likely to confer high affinity for pKA. The mutation L34S may not be needed. Other mutations that might foster pKA binding include Y21W, Y21F, Q32E, L34T, L34I, and E39G.

DKI-3.2.1 (Table 2) is derived from **Human TFPI-2 domain 2**. This parental domain contains insertions after residue 9 (one residue) and 42 (two residues). The mutations E15R, G16A, S17A, T18H,E19P, K32T, and F34V are intended to confer affinity for pKA. If one needs a pKA inhibitor based on TFPI domain 2, a preferred route is to make a library of domains allowing the substitutions given and many others and then select binders.

DKI-3.3.1 (Table 2) is derived from **human TFPI-2, domain 3**. The substitutions L13H, S15R, and N17A are likely to confer high affinity for pKA. Other mutations that might foster pKA binding include D10E, T19Q, Y21W, T36G, and G39E.

DKI-4.1.1(Table 2) is from **human ITI-K1** by assertion of S10D, M15R, M17A, T18H, Q34S, and M39G. The mutations M39G and Q34V may not be necessary. Other mutations that should foster pKA binding include: G16A, M17N, S19Q, Y21W, and Y21F.

DKI-4.2.1(Table 2) is from **human ITI-K2** through the mutations V10D, R11D, F17A, I18H, V31E, L32E, P34S, and Q39E. The mutations V31E, L32E, and Q39E might not be necessary. Other mutation that should foster pKA binding include: V10E, Q19P, L20R, W21F, P34I, and Q39G. DKI-4.2.2 has eight mutations: V10D, R11D, F17A, I18H, L20R, V31E, L32E, and P34S.

DKI-5.1 is derived from human APP-I (also known as Protease Nexin-II) by mutations M17A, I18H, S19P, A31E, and P32E and is likely to be a potent pKA inhibitor. The mutations S19P, A31E, and P32E many not be needed. Other mutations that might foster pKA binding include T11D.

DKI-6.1 is derived from the **HKI B9 KuDom** (NORR93) by the five substitutions: K11D,

Q15R, T16A, M17A, M18H, T19P, and L32E. DKI-6.1 is likely to be a potent pKA inhibitor. The mutations L32E, and T19P might not be needed.

Although BPTI is not an especially good pKA inhibitor, it could be made into one. DKI-7.1 is derived from BPTI by the mutations Y10E, K15R, R17A, RI18H, I19P, Q31E, T32E, and R39E which is likely to increase the affinity for pKA. The mutations Y10E, K15R, I19P, Q31E, T32E, and R39E may not be needed; the really important mutations are R17A and RI18H.

MODIFICARION OF KUNITZ DOMAINS

KuDoms are quite small; if this should cause a pharmacological problem, such as excessively quick elimination from circulation, two or more such domains may be joined. A preferred linker is a sequence of one or more amino acids. A preferred linker is one found between repeated domains of a human protein, especially the linkers found in human BPTI homologues, one of which has two domains (BALD85, ALBR83a, ALBR83b) and another of which has three (WUNT88). Peptide linkers have the advantage that the entire protein may then be expressed by recombinant DNA techniques. It is also possible to use a nonpeptidyl linker, such as one of those commonly used to form immunogenic conjugates. An alternative means of increasing the serum residence of a BPTI-like KuDom is to link it to polyethyleneglycol, so called PEGylation (DAVI79).

WAYS TO IMPROVE SPECIFICITY OF, FOR EXAMPLE, KKII/3#7, KK2/#11, AND KK2/#13 FOR PLASMA KALLIKREIN:

Because we have made a large part of the surface of KKII/3#6, KK2/#11, and KK2/#13 complementary to the surface of pKA, R_{15} is not essential for specific binding to pKA. Many of the enzymes in the clotting and fibrinolytic pathways cut preferentially after Arg or Lys. Not having a basic residue at the P1 position may give rise to greater specificity. The variant KKII/3#7-K15A (shown in Table 27), having an ALA at P1, is likely to be a good pKA inhibitor and may have higher specificity for pKA relative to other proteases than doesKKII/3#7. The affinity of KKII/3#7-K15A for pKA is likely to be less than the affinity of KKII/3#7 for pKA, but the loss of affinity for other Arg/Lys-preferring enzymes is likely to be greater and, in many applications, specificity is more important than affinity. Other mutants that are likely to have good affinity and very high specificity include KK2/#13-R15A and KK2/#11-R15S. This approach could be applied to other high-affinity pKA inhibitors.

MODE OF PRODUCTION

The proteins of this invention may be produced by any conventional technique, including

(a) nonbiological synthesis by sequential coupling of component amino acids,

(b) production by recombinant DNA techniques in a suitable host cell, and

(c) removal of undesired sequences from LACI and coupling of synthetic replacement sequences

The proteins disclosed herein are preferably produced, recombinantly, in a suitable host, such as bacteria from the genera Bacillus, Escherichia, Salmonella, Erwinia, and yeasts from the genera Hansenula, Kluyveromyces, Pichia, Rhinosporidium, Saccharomyces, and Schizosaccharomyces, or cultured mammalian cells such as COS-1. The more preferred hosts are microorganisms of the species *Pichia pastoris*, *Bacillus subtilis*, *Bacillus brevis*, *Saccharomyces cerevisiae*, *Escherichia coli* and *Yarrowia lipolytica*. Any promoter, regulatable or constitutive, which is functional in the host may be used to control gene expression.

Preferably the proteins are secreted. Most preferably, the proteins are obtained from conditioned medium. It is not required that the proteins described herein be secreted. Secretion is the preferred route because proteins are more likely to fold correctly, can be produced in conditioned medium with few contaminants, and are less likely to be toxic to host cells. Secretion is not required.

Unless there is a specific reason to include glycogroups, we prefer proteins designed to lack N-linked glycosylation sites to reduce potential for antigenicity of glycogroups and so that equivalent proteins can be expressed in a wide variety of organisms including: 1) *E. coli*, 2) *B. subtilis*, 3) *P. pastoris*, 4) *S. cerevisiae*, and 5) mammalian cells.

Several means exist for reducing the problem of host cells producing proteases that degrade the recombinant product; see, *inter alia* BANE90 and BANE91. VAND92 reports that overexpression of the *B. subtilis* signal peptidase in *E. coli*. leads to increased expression of a heterologous fusion protein. ANBA88 reports that addition of PMSF (a serine proteases inhibitor) to the culture medium improved the yield of a fusion protein.

Other factors that may affect production of these and other proteins disclosed herein include: 1) codon usage (optimizing codons for the host is preferred), 2) signal sequence, 3) amino-acid sequence at intended processing sites, presence and localization of processing

enzymes, deletion, mutation, or inhibition of various enzymes that might alter or degrade the engineered product and mutations that make the host more permissive in secretion (permissive secretion hosts are preferred).

Reference works on the general principles of recombinant DNA technology include Watson *et al.*, **Molecular Biology of the Gene**, Volumes I and II, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, CA (1987); Darnell *et al.*, **Molecular Cell Biology**, Scientific American Books, Inc., New York, N.Y. (1986); Lewin, **Genes II**, John Wiley & Sons, New York, N.Y. (1985); Old, *et al.*, **Principles of Gene Manipulation: An Introduction to Genetic Engineering**, 2d edition, University of California Press, Berkeley, CA (1981); Sambrook *et al.*, **Molecular Cloning: A Laboratory Manual**, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989); and Ausubel *et al*, **Current Protocols in Molecular Biology**, Wiley Interscience, NY, (1987, 1992). These references are herein entirely incorporated by reference as are the references cited therein.

PREPARATION OF PEPTIDES

Chemical polypeptide synthesis is a rapidly evolving area in the art, and methods of solid phase polypeptide synthesis are well-described in the following references, hereby entirely incorporated by reference: (Merrifield, *J Amer Chem Soc* **85**:2149-2154 (1963); Merrifield, *Science* **232**:341-347 (1986); Wade *et al.*, *Biopolymers* **25**:S21-S37 (1986); Fields, *Int J Polypeptide Prot Res* **35**:161 (1990); MilliGen Report Nos. 2 and 2a, Millipore Corporation, Bedford, MA, 1987) Ausubel *et al, supra*, and Sambrook *et al, supra.* Tan and Kaiser (*Biochemistry*, 1977, **16**:1531-41) synthesized BPTI and a homologue eighteen years ago.

As is known in the art, such methods involve blocking or protecting reactive functional groups, such as free amino, carboxyl and thio groups. After polypeptide bond formation, the protective groups are removed. Thus, the addition of each amino acid residue requires several reaction steps for protecting and deprotecting. Current methods utilize solid phase synthesis, wherein the C-terminal amino acid is covalently linked to an insoluble resin particles that can be filtered. Reactants are removed by washing the resin particles with appropriate solvents using an automated machine. Various methods, inclulding the "tBoc" method and the "Fmoc" method are well known in the art. See, *inter alia*, Atherton *et al.*, *J Chem Soc Perkin Trans* **1**:538-546 (1981) and Sheppard *et al.*, *Int J Polypeptide Prot Res* **20**:451-454 (1982).

ASSAYS FOR PLASMA KALLIKREIN BINDING AND INHIBITION

Any suitable method may be used to test the compounds of this invention. Scatchard (*Ann NY*

Acad Sci (1949) **51**:660-669) described a classical method of measuring and analyzing binding which is applicable to protein binding. This method requires relatively pure protein and the ability to distinguish bound protein from unbound.

A second appropriate method of measuring K_D is to measure the inhibitory activity against the enzyme. If the K_D to be measured is in the 1 nM to 1 μM range, this method requires chromogenic or fluorogenic substrates and tens of micrograms to milligrams of relatively pure inhibitor. For the proteins of this invention, having K_D in the range 5 nM to 50 pM, nanograms to micrograms of inhibitor suffice. When using this method, the competition between the inhibitor and the enzyme substrate can give a measured K_i that is higher than the true K_i. Measurement reported here are not so corrected because the correction would be very small and the any correction would reduce the K_i. Here, we use the measured K_i as a direct measure of K_D.

A third method of determining the affinity of a protein for a second material is to have the protein displayed on a genetic package, such as M13, and measure the ability of the protein to adhere to the immobilized "second material". This method is highly sensitive because the genetic packages can be amplified. We obtain at least semiquantitative values for the binding constants by use of a pH step gradient. Inhibitors of known affinity for the protease are used to establish standard profiles against which other phage-displayed inhibitors are judged. Any other suitable method of measuring protein binding may be used.

Preferably, the proteins of this invention have a K_D for pKA of at most about 5nM, more preferably at most about 300 pM, and most preferably 100 pM or less. Preferably, the binding is inhibitory so that K_i is the same as K_D. The K_i of KKII/3#6 is about 300 pM and the K_is of KK2/#11 and KK2/#13 are less than 300 pM and probably less than 100 pM.

PHARMACEUTICAL METHODS AND PREPARATIONS

The preferred subject of this invention is a mammal. The invention is particularly useful in the treatment of humans, but is suitable for veternary applications too.

Herein, "protection" includes "prevention", "suppression", and "treatment". "Prevention" involves administration of drug prior to the induction of disease. "Suppression" involves administration of drug prior to the clinical appearance of disease. "Treatment" involves administration of drug after the appearance of disease.

In human and veterinary medicine, it may not be possible to distinguish between "preventing" and "suppressing" since the inductive event(s) may be unknown or latent, or the patient is not ascertained until after the occurrence of the inductive event(s). We use the term

"prophylaxis" as distinct from "treatment" to encompass "preventing" and "suppressing". Herein, "protection" includes "prophylaxis". Protection need not by absolute to be useful.

Proteins of this invention may be administered, by any means, systemically or topically, to protect a subject against a disease or adverse condition. For example, administration of such a composition may be by any parenteral route, by bolus injection or by gradual perfusion. Alternatively, or concurrently, administration may be by the oral route. A suitable regimen comprises administration of an effective amount of the protein, administered as a single dose or as several doses over a period of hours, days, months, or years.

The suitable dosage of a protein of this invention may depend on the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the desired effect. However, the most preferred dosage can be tailored to the individual subject, as is understood and determinable by one of skill in the art, without undue experimentation by adjustment of the dose in ways known in the art.

For methods of preclinical and clinical testing of drugs, including proteins, see, e.g., Berkow *et al*, eds., **The Merck Manual**, 15th edition, Merck and Co., Rahway, N.J., 1987; Goodman *et al.*, eds., **Goodman and Gilman's The Pharmacological Basis of Therapeutics**, 8th edition, Pergamon Press, Inc., Elmsford, N.Y., (1990); **Avery's Drug Treatment: Principles and Practice of Clinical Pharmacology and Therapeutics**, 3rd edition, ADIS Press, LTD., Williams and Wilkins, Baltimore, MD. (1987), Ebadi, **Pharmacology**, Little, Brown and Co., Boston, (1985), which references and references cited there are hereby incorporated by reference.

In addition to a protein here disclosed, a pharmaceutical composition may contain pharmaceutically acceptable carriers, excipients, or auxiliaries. See, *e.g.*, Berker, *supra*, Goodman, *supra*, Avery, *supra* and Ebadi, *supra*.

IN VITRO DIAGNOSTIC METHODS AND REAGENTS

Proteins of this invention may be applied *in vitro* to any suitable sample that might contain plasma kallikrein to measure the pKA present. To do so, the assay must include a Signal Producing System (SPS) providing a detectable signal that depends on the amount of pKA present. The signal may be detected visually or instrumentally. Possible signals include production of colored, fluorescent, or luminescent products, alteration of the characteristics of absorption or emission of radiation by an assay component or product, and precipitation or agglutination of a component or product.

The component of the SPS most intimately associated with the diagnostic reagent is called

the "label". A label may be, *e.g.*, a radioisotope, a fluorophore, an enzyme, a co-enzyme, an enzyme substrate, an electron-dense compound, or an agglutinable particle. A radioactive isotope can be detected by use of, for example, a γ counter or a scintillation counter or by autoradiography. Isotopes which are particularly useful are ^{3}H, ^{125}I, ^{131}I, ^{35}S, ^{14}C, and, preferably, ^{125}I. It is also possible to label a compound with a fluorescent compound. When the fluorescently labeled compound is exposed to light of the proper wave length, its presence can be detected. Among the most commonly used fluorescent labelling compounds are fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, *o*-phthaldehyde, and fluorescamine. Alternatively, fluorescence-emitting metals, such as ^{125}Eu or other lanthanide, may be attached to the binding protein using such metal chelating groups as diethylenetriaminepentaacetic acid or ethylenediamine-tetraacetic acid. The proteins also can be detectably labeled by coupling to a chemiluminescent compound, such as luminol, isolumino, theromatic acridinium ester, imidazole, acridinium salt, and oxalate ester. Likewise, a bioluminescent compound, such as luciferin, luciferase and aequorin, may be used to label the binding protein. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Enzyme labels, such as horseradish peroxidase and alkaline phosphatase, are preferred.

There are two basic types of assays: heterogeneous and homogeneous. In heterogeneous assays, binding of the affinity molecule to analyte **does not** affect the label; thus, to determine the amount of analyte, bound label must be separated from free label. In homogeneous assays, the interaction **does** affect the activity of the label, and analyte can be measured without separation.

In general, a kallikrein-binding protein (KBP) may be used diagnostically in the same way that an anti-pKA antibody is used. Thus, depending on the assay format, it may be used to assay pKA, or, by competitive inhibition, other substances which bind pKA.

The sample will normally be a biological fluid, such as blood, urine, lymph, semen, milk, or cerebrospinal fluid, or a derivative thereof, or a biological tissue, *e.g.*, a tissue section or homogenate. The sample could be anything. If the sample is a biological fluid or tissue, it may be taken from a human or other mammal, vertebrate or animal, or from a plant. The preferred sample is blood, or a fraction or derivative thereof.

In one embodiment, the pKA-binding protein (KBP) is immobilized, and pKA in the sample is allowed to compete with a known quantity of a labeled or specifically labelable pKA analogue. The "pKA analogue" is a molecule capable of competing with pKA for binding to the KBP, which includes pKA itself. It may be labeled already, or it may be labeled subsequently by

specifically binding the label to a moiety differentiating the pKA analogue from pKA. The phases are separated, and the labeled pKA analogue in one phase is quantified.

In a "sandwich assay", both an insolubilized pKA-binding agent (KBA), and a labeled KBA are employed. The pKA analyte is captured by the insolubilized KBA and is tagged by the labeled KBA, forming a tertiary complex. The reagents may be added to the sample in any order. The KBAs may be the same or different, and only one KBA need be a KBP according to this invention (the other may be, *e.g.*, an antibody). The amount of labeled KBA in the tertiary complex is directly proportional to the amount of pKA in the sample.

The two embodiments described above are both heterogeneous assays. A homogeneous assay requires only that the label be affected by the binding of the KBP to pKA. The pKA analyte may act as its own label if a pKA inhibitor is used as a diagnostic reagent.

A label may be conjugated, directly or indirectly (*e.g.*, through a labeled anti-KBP antibody), covalently (*e.g.*, with SPDP) or noncovalently, to the pKA-binding protein, to produce a diagnostic reagent. Similarly, the pKA binding protein may be conjugated to a solid phase support to form a solid phase ("capture") diagnostic reagent. Suitable supports include glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amylases, and magnetite. The carrier can be soluble to some extent or insoluble for the purposes of this invention. The support material may have any structure so long as the coupled molecule is capable of binding pKA.

IN VIVO DIAGNOSTIC USES

A Kunitz domain that binds very tightly to pKA can be used for *in vivo* imaging. Diagnostic imaging of disease foci was considered one of the largest commercial opportunities for monoclonal antibodies, but this opportunity has not been achieved. Despite considerable effort, only two monoclonal antibody-based imaging agents have been approved. The disappointing results obtained with monoclonal antibodies is due in large measure to:

i) Inadequate affinity and/or specificity;

ii) Poor penetration to target sites;

iii) Slow clearance from nontarget sites;

iv) Immunogenicity (most are murine); and

v) High production cost and poor stability.

These limitations have led most in the diagnostic imaging field to begin to develop peptide-based imaging agents. While potentially solving the problems of poor penetration and slow clearance, peptide-based imaging agents are unlikely to possess adequate affinity, specificity and *in vivo*

stability to be useful in most applications.

Engineered proteins are uniquely suited to the requirements for an imaging agent. In particular the extraordinary affinity and specificity that is obtainable by engineering small, stable, human-origin protein domains having known *in vivo* clearance rates and mechanisms combine to provide earlier, more reliable results, less toxicity/side effects, lower production and storage cost, and greater convenience of label preparation. Indeed, it should be possible to achieve the goal of realtime imaging with engineered protein imaging agents. Thus, a Kallikrein-binding protein, e.g., KKII/3#6, KK2/#11, and KK2/#13 may be used for localizing sites of excessive pKA activity.

Radio-labelled binding protein may be administered to the human or animal subject. Administration is typically by injection, *e.g.*, intravenous or arterial or other means of administration in a quantity sufficient to permit subsequent dynamic and/or static imaging using suitable radio-detecting devices. The dosage is the smallest amount capable of providing a diagnostically effective image, and may be determined by means conventional in the art, using known radio-imaging agents as guides.

Typically, the imaging is carried out on the whole body of the subject, or on that portion of the body or organ relevant to the condition or disease under study. The radio-labelled binding protein has accumulated. The amount of radio-labelled binding protein accumulated at a given point in time in relevant target organs can then be quantified.

A particularly suitable radio-detecting device is a scintillation camera, such as a γ camera. The detection device in the camera senses and records (and optional digitizes) the radioactive decay. Digitized information can be analyzed in any suitable way, many of which are known in the art. For example, a time-activity analysis can illustrate uptake through clearance of the radio-labelled binding protein by the target organs with time.

Various factors are taken into consideration in picking an appropriate radioisotope. The isotope is picked: to allow good quality resolution upon imaging, to be safe for diagnostic use in humans and animals, and, preferably, to have a short half-life so as to decrease the amount of radiation received by the body. The radioisotope used should preferably be pharmacologically inert, and the quantities administered should not have substantial physiological effect. The binding protein may be radio-labelled with different isotopes of iodine, for example ^{123}I, ^{125}I, or ^{131}I (see, for example, U.S. Patent 4,609,725). The amount of labeling must be suitably monitored.

In applications to human subjects, it may be desirable to use radioisotopes other than ^{125}I

for labelling to decrease the total dosimetry exposure of the body and to optimize the detectability of the labelled molecule. Considering ready clinical availability for use in humans, preferred radio-labels include: ^{99m}Tc, ^{67}Ga, ^{68}Ga, ^{90}Y, ^{111}In, ^{113m}In, ^{123}I, ^{186}Re, ^{188}Re or ^{211}At. Radio-labelled protein may be prepared by various methods. These include radio-halogenation by the chloramine-T or lactoperoxidase method and subsequent purification by high pressure liquid chromatography, for example, see Gutkowska *et al* in "Endocrinology and Metabolism Clinics of America: (1987) 16 (1):183. Other methods of radio-labelling can be used, such as IODOBEADS™.

A radio-labelled protein may be administered by any means that enables the active agent to reach the agent's site of action in a mammal. Because proteins are subject to digestion when administered orally, parenteral administration, *i.e.*, intravenous subcutaneous, intramuscular, would ordinarily be used to optimize absorption.

High-affinity, high-specificity inhibitors are also useful for *in vitro* diagnostics of excess human pKA activity.

Other Uses

The kallikrein-binding proteins of this invention may also be used to purify kallikrein from a fluid, *e.g.*, blood. For this, the KBP is preferably immobilized on a support. Such supports, include those already mentioned as useful in preparing solid phase diagnostic reagents.

Proteins can be used as molecular weight markers for reference in the separation or purification of proteins. Proteins may need to be denatured to serve as molecular weight markers. A second general utility for proteins is the use of hydrolyzed protein as a nutrient source. Proteins may also be used to increase the viscosity of a solution.

The proteins of this invention may be used for any of the foregoing purposes, as well as for therapeutic and diagnostic purposes as discussed further earlier in this specification.

EXAMPLE 1: CONSTRUCTION OF FIRST LACI-K1 LIBRARY

A synthetic oligonucleotide duplex having *Nsi*I- and *Mlu*I-compatible ends was cloned into a parental vector (LACI:III) previously cleaved with the above two enzymes. The resultant ligated material was transfected by electroporation into XLIMR (F^-) *Escherichia coli* strain and plated on Amp plates to obtain phage-generating Ap^R colonies. The variegation scheme for Phase 1 focuses on the P1 region, and affected residues 13, 16, 17, 18 and 19. It allowed for 6.6×10^5 different DNA sequences (3.1×10^5 different protein sequences). The library obtained consisted of 1.4×10^6 independent cfu's which is approximately a two fold representation of the whole

library. The phage stock generated from this plating gave a total titer of 1.4×10^{13} pfu's in about 3.9 ml, with each independent clone being represented, on average, 1×10^7 in total and 2.6×10^6 times per ml of phage stock.

To allow for variegation of residues 31, 32, 34 and 39 (phase II), synthetic oligonucleotide duplexes with *Mlu*I- and *Bst*EII- compatible ends were cloned into previously cleaved R_f DNA derived from one of the following

i) the parental construction,

ii) the phase I library, or

iii) display phage selected from the first phase binding to a given target.

The variegation scheme for phase II allows for 4096 different DNA sequences (1600 different protein sequences) due to alterations at residues 31, 32, 34 and 39. The final phase II variegation is dependent upon the level of variegation remaining following the three rounds of binding and elution with a given target in phase I.

The combined possible variegation for both phases equals 2.7×10^8 different DNA sequences or 5.0×10^7 different protein sequences. When previously selected display phage are used as the origin of R_f DNA for the phase II variegation, the final level of variegation is probably in the range of 10^5 to 10^6.

Example 2: Screening of LACI (K1) Library for Binding to Kallikrein

The overall scheme for selecting a LACI-K1 variant to bind to a given protease involves incubation of the phage-display library with the kallikrein-beads of interest in a buffered solution (PBS containing 1 mg/ml BSA) followed by washing away the unbound and poorly retained display-phage variant with PBS containing 0.1% Tween 20. Kallikrein beads were made by coupling human plasma Kallikrein (Calbiochem, San Diego, CA, # 420302) to agarose beads using Reactigel (6x) (Pierce, Rockford, Il, #202606). The more strongly bound display-phage are eluted with a low pH elution buffer, typically citrate buffer (pH 2.0) containing 1 mg/ml BSA, which is immediately neutralized with Tris buffer to pH 7.5. This process constitutes a single round of selection.

The neutralized eluted display-phage can be either used:

i) to inoculate an F^+ strain of *E. coli* to generate a new display-phage stock, to be used for subsequent rounds of selection (so-called conventional screening), or

ii) be used directly for another immediate round of selection with the protease beads (so-called quick screening).

Typically, three rounds of either method, or a combination of the two, are performed to give rise to the final selected display-phage from which a representative number are sequenced and analyzed for binding properties either as pools of display-phage or as individual clones.

Two phases of selection were performed, each consisting of three rounds of binding and elution. Phase I selection used the phase I library (variegated residues 13, 16, 17, 18, and 19) which went through three rounds of binding and elution against a target protease giving rise to a subpopulation of clones. The R_f DNA derived from this selected subpopulation was used to generate the Phase II library (addition of variegated residues 31, 32, 34 and 39). The 1.8×10^7 independent transformants were obtained for each of the phase II libraries. The phase II libraries underwent three further rounds of binding and elution with the same target protease giving rise to the final selectants.

Following two phases of selection against human plasma kallikrein-agarose beads a number (10) of the final selection display-phage were sequenced. The amino-acid sequences are shown in Table 2, entries KBPcon1 through KKII/3#C.

Table 23 shows that KkII/3(D) is a highly specific inhibitor of human Kallikrein. Phage that display the LACI-K1 derivative KkII/3(D) bind to Kallikrein beads at least 50-times more than it binds to other protease targets.

Preliminary measurements indicate that KKII/3#6 is a potent inhibitor of pKA with K_i probably less than 500 pM.

EXPRESSION, PURIFICATION AND KINETIC ANALYSIS.

The three isolates KKII/3#6, KK2/#11, and KK2/#13 were recloned into a yeast expression vector. The yeast expression vector is derived from pMFalpha8 (KURJ82 and MIYA85). The LACI variant genes were fused to part of the matα1 gene, generating a hybrid gene consisting of the matα1 promoter-signal peptide and leader sequence-fused to the LACI variant. The cloning site is shown in Table 24. Note that the correctly processed LACI-K1 variant protein should be as detailed in Table 2 with the addition of residues glu-ala-ala-glu to the N-terminal met (residue 1 in Table 2). Expression in *S. cerevisiae* gave acceptable yield typical of this system. Yeast-expressed LACI (kunitz domain 1), BPTI and LACI variants: KKII/3#6, KK2/#11, and KK2/#13 were purified by affinity chromatography using trypsin-agarose beads.

For larger-scale production, *Pichia pastoris* is a preferred host. The most preferred production sytem in *P. pastoris* is the alcohol oxidase system. Others have produced a number

of proteins in the yeast *Pichia pastoris*. For example, Vedvick *et al.* (VEDV91) and Wagner *et al.* (WAGN92) produced aprotinin from the alcohol oxidase promoter with induction by methanol as a secreted protein in the culture medium at ≈1 mg/ml. Gregg et al. (GREG93) have reviewed production of a number of proteins in P. pastoris. Table 1 of GREG93 shows proteins that have been produced in *P. pastoris* and the yields.

All references, including those to U.S. and foreign patents or patent applications, and to nonpatent disclosures, are hereby incorporated by reference in their entirety.

TABLE 1: Sequence of whole LACI:

1 5 5 5 5 5

1 MIYTMKKVHA LWASVCLLLN LAPAPLNAds eedeehtiit dtelpplklM
51 HSFCAFKADD GPCKAIMKRF FFNIFTRQCE EFIYGGCEGN QNRFESLEEC
101 KKMCTRDnan riikttlqqe kpdfcfleed pgicrgyitr yfynnqtkqc
151 erfkyggclg nmnnfetlee cknicedgpn gfqvdnygtq lnavnnsltp
201 qstkvpslfe fh**gpswcltp adrglcrane nrfyynsvig kcrpfkysgc**
251 **ggnennftsk qeclrackkg** fiqriskggl iktkrkrkkq rvkiayeeif
301 vknm (SEQ ID NO. 18)

The signal sequence (1-28) is uppercase and underscored
LACI-K1 is uppercase
LACI-K2 is underscored
LACI-K3 is bold

TABLE 2 is below.

Table 3: Summary of first selection of LACI-K1 domains for binding to pKA.

BPTI #	(Lac I)	Library Residues	Preferred Residues
13	P	LHPR	HP
16	A	AG	AG
17	I	FYLHINA SCPRTVD G	NSA
18	M	all	HL
19	K	LWQMKAG SPRTVE	QLP
31	E	EQ	E
32	E	EQ	EQ
34	I	all	STI
39	E	all	GEA

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
BPTI	RPDFCLEPPYTGPCKARIIRYFYNAKAGLCQTFVYGGCRAKRNNFKSAEDCMRTCGGA	SEQ ID NO. 2
LACI-K1	mhsfcafkaddgpckaimkrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 3
KBPcon1	mhsfcafkaddgHckaNHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 4
KKII/3#1	mhsfcafkaddgHckASLPrfffniftrqcEEfIyggcEgnqnrfesleeckkmctrd	SEQ ID NO. 5
KKII/3#2	mhsfcafkaddgPckANHLrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 6
KKII/3#3	mhsfcafkaddgHckANHQrfffniftrqcEEfTyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 7
KKII/3#4	mhsfcafkaddgHckANHQrfffniftrqcEQfTyggcAgnqnrfesleeckkmctrd	SEQ ID NO. 8
KKII/3#5	mhsfcafkaddgHckASLPrfffniftrqcEEfIyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 9
KKII/3#6	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 10
KKII/3#7	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 11
KKII/3#8	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 12
KKII/3#9	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 13
KKII/3#10	mhsfcafkaddgHckGAHLrfffniftrqcEEfIyggcEgnqnrfesleeckkmctrd	SEQ ID NO. 14
KKI/3(a)	mhsfcafkaddgRckGAHLrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 15

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
KKI/3(b)	mhsfcafkaddgPckAIHLrfffniftrqceefiyggcegnqnrfesleeckkmctrd	SEQ ID NO. 16
KKII/3#C	mhsfcafkaddgHckANHQrfffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 17
KK2/#13	mhsfcafkaDGgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 19
KK2/#14	mhsfcafkaDGgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 20
KK2/#5	mhsfcafkaDDgPcRAAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 21
KK2/#11	mhsfcafkaDDgPcRAAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 22
KK2/#1	mhsfcafkaDVgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 23
KK2/#4	mhsfcafkaDVgRcRGAQPrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 24
KK2/#6	mhsfcafkaDDgScRAAHLrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 25
KK2/#10	mhsfcafkaEGgScRAAHQrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 26
KK2/#8	mhsfcafkaDDgPcRGAHLrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 27
KK2/#3	mhsfcafkaDDgHcRGALPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 28
KK2/#9	mhsfcafkaDSgNcRGNLPrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 29
KK2/#7	mhsfcafkaDSgRcRGNHQrFffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 30

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
KK2/#12	mhsfcafkaDGgRcRAIQPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 31
KK2con1	mhsfcafkaDDgRcRGAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd	SEQ ID NO. 32
Human LACI-K2	KPDFCFLEEDPGICRGYITRYFYNNQTKQCERFKYGGCLGNMNNFETLEECKNICEDG	SEQ ID NO. 33
DKI-1.2.1	kpdfcfleedGgRcrgAHPrWfynnqtkqceEfSyggcGgnmnnfetleecknicedg	SEQ ID NO. 34
Human LACI-K3	GPSWCLTPADRGLCRANENRFYYNSVIGKCRPFKYSGCGGNENNFTSKQECLRACKKG	SEQ ID NO. 35
DKI-1.3.1	gpswcltpadDgPcraAHPrfyynsvigkcEpfSysgcggnennftskqeclrackkg	SEQ ID NO. 36
Human collagen α3 KuDom	ETDICKLPKDEGTCRDFILKWYYDPNTKSCARFWYGGCGGNENKFGSQKECEKVCAPV	SEQ ID NO. 37
DKI-2.1	etdicklpkdegtcrAAHlkwyydpntkscaEfSyggcggnenkfgsqkecekvcapv	SEQ ID NO. 38
TFPI-2 DOMAIN 1	NAEICLLPLDYGPCRALLLRYYYDRYTQSCRQFLYGGCEGNANNFYTWEACDDACWRI	SEQ ID NO. 39
DKI-3.1.1	naeicllpldGgpcraAHlryyydrytqscEqfSyggcegnannfytweacddacwri	SEQ ID NO. 40
tfpi-2 DOMAIN 2	VPKVCRLQVS- VDDQCEGSTEKYFFNLSSMTCEKFFSGGCHRNR- IENRFPDEATCMGFCAPK	SEQ ID NO. 41

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.

Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
DKI-3.2.1	vpkvcrlqvs- vddqcRAAHPkyffnlssmtceEffsggchrnr- ienrfpdeatcmgfcapk	SEQ ID NO. 42
TFPI-2 DOMAIN 3	IPSFCYSPKDEGLCSANVTRYYFNPRYRTCDAFTYTGCGGNDNNFVSREDCKRACAKA	SEQ ID NO. 43
DKI-3.3.1	ipsfcyspkdegHcRaAHQryyfnpryrtcdaftytgcggndnnfvsredckracaka	SEQ ID NO. 44
HUMAN ITI-K1	KEDSCQLGYSAGPCMGMTSRYFYNGTSMACETFQYGGCMGNGNNFVTEKECLQTCRTV	SEQ ID NO. 45
DKI-4.1.1	kedscqlgyDagpcRgAHPryfyngtsmacetfSyggcGgngnnfvtekeclqtcrtv	SEQ ID NO. 46
Human ITI-K2	TVAACNLPIVRGPCRAFIQLWAFDAVKGKCVLFPYGGCQGNGNKFYSEKECREYCGVP	SEQ ID NO. 47
DKI-4.2.1	tvaacnlpiDDgpcraAHqlwafdavkgkcEEfSyggcEgngnkfysekecreycgvp	SEQ ID NO. 48
DKI-4.2.2	tvaacnlpiDDgpcraAHqRwafdavkgkcEEfSyggcqgngnkfysekecreycgvp	SEQ ID NO. 49
HUMAN PROTEASE NEXIN-II	VREVCSEQAETGPCRAMISRWYFDVTEGKCAPFFYGGCGGNRNNFDTEEYCMAVCGSA	SEQ ID NO. 50
DKI-5.1	vrevcseqaetgpcraAHPrwyfdvtegkcEEfSyggcggnrnnfdteeycmavcgsa	SEQ ID NO. 51
HKI B9 domain	LPNVCAFPMEKGPCQTYMTRWFFNFETGECELFAYGGCGGNSNNFLRKEKCEKFCKFT	SEQ ID NO. 53
DKI-6.1	lpnvcafpmeDgpcRAAHPrwffnfetgeceEfayggcggnsnnflrkekcekfckft	SEQ ID NO. 54

Table 2: Sequences of Kunitz domains, some of which inhibit human pKA.		
Ident	Amino-acid sequence 1111111111222222222233333333334444444444555555555 12345678901234567890123456789012345678901234567890123456789012345678	SEQ ID NO.
DKI-7.1	rpdfcleppEtgpcRaAHPryfynakaglcEEfvyggcGakrnnfksaedcmrtcgga	SEQ ID NO. 55

Table 3 is above.

TABLE 8: Binding Data for Selected Kallikrein-binding Display-Phage.

Display-Phage(a)	Fraction Bound(b)	Relative Binding(c)
LACI	4.2×10^{-6}	1.0
BPTI	2.5×10^{-5}	6.0
KKI/3(a)	3.2×10^{-3}	761
KKI/3(b)	2.2×10^{-3}	524
KKII/3#5	3.9×10^{-3}	928
KKII/3#6	8.7×10^{-3}	2071

(a) Clonal isolates of display-phage. LACI-K1 is the parental molecule, BPTI (bovine pancreatic trypsin inhibitor) is a control and KKII/3 (5 and 6) and KKI/3(a and b) were selected by binding to the target protease, kallikrein.

(b) The number of pfu's eluted after a binding experiment as a fraction of the input number (10^{10} pfu's).

(c) Fraction bound relative to the parental display-phage, LACI-K1.

Table 9: Conservative and Semiconservative substitutions

Initial AA type	Category	Conservative substitution	Semi-conservative substitution
A	Small non-polar or slightly polar	G, S, T	N, V, P, (C)
C	free SH	A, M, L, V, I	F, G
	disulfide	nothing	nothing
D	acidic, hydrophilic	E, N, S, T, Q	K, R, H, A
E	acidic, hydrophilic	D, Q, S, T, N	K, R, H, A
F	aromatic	W, Y, H, L, M	I, V, (C)
G	Gly-only conformation	nothing	nothing
	"normal" conformation	A, S, N, T	D, E, H, I, K, L, M, Q, R, V
H	amphoteric aromatic	Y, F, K, R	L, M, A, (C)
I	aliphatic, branched β carbon	V, L, M, A	F, Y, W, G (C)
K	basic	R, H	Q, N, S, T, D, E, A
L	aliphatic	M, I, V, A	F, Y, W, H, (C)
M	hydrophobic	L, I, V, A	Q, F, Y, W, (C), (R), (K), (E)
N	non-polar hydrophilic	S, T, (D), Q, A, G, (E)	K, R
P	inflexible	V, I	A, (C), (D), (E), F, H, (K), L, M, N, Q, (R), S, T, W, Y
Q	aliphatic plus amide	N, E, A, S, T, D	M, L, K, R
R	basic	K, Q, H	S, T, E, D, A,
S	hydrophilic	A, T, G, N	D, E, R, K

Table 9: Conservative and Semiconservative substitutions

Initial AA type	Category	Conservative substitution	Semi-conservative substitution
T	hydrophilic	A, S, G, N, V	D, E, R, K, I
V	aliphatic, branched β carbon	I, L, M, A, T	P, (C)
W	aromatic	F, Y, H	L, M, I, V, (C)
Y	aromatic	F, W, H	L, M, I, V, (C)

Changing from A, F, H, I, L, M, P, V, W, or Y to C is semiconservative if the new cysteine remains as a free thiol.

Changing from M to E, R, K is semiconservative if the ionic tip of the new side group can reach the protein surface while the methylene groups make hydrophobic contacts.

Changing from P to one of K, R, E, or D is semiconservative if the side group is on or near the surface of the protein.

Table 14: Definition of a Kunitz Domain (SEQ ID NO. 52)
1 2 3 4 5 12345678901234567890123456789012345678901234567890123456789012345678 xxxxCxxxxxxGxCxxxxxxXXXxxxxxxxCxxFxXXGCxXxxXxXxxxxxCxxxCxxx
Where: *X1, X2, X3, X4, X58, X57, and X56 may be absent,*
X21 = Phe, Tyr, Trp,
X22 = Tyr or Phe,
X23 = Tyr or Phe,
X35 = Tyr or Trp,
X36 = Gly or Ser,
X40 = Gly or Ala,
X43 = Asn or Gly, and
X45 = Phe or Tyr

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
10	**Asp, Glu**	Ala, Gly, Ser, Thr	Lys, Asn, (Arg, Cys, Phe, His, Ile, Leu, Met, Pro, Gln, Val, Trp, Tyr)
11	**Asp, Gly, Ser, Val**	Glu, Leu, Met, [Asn, Ile, Ala, Thr]	(Cys, Phe, His, Lys, Pro, Gln, Arg, Trp, Tyr)
12	**Gly**	(Other amino acids ONLY if C_{14}-C_{38} disulfide replaced by other amino acids.)	

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
13	**Arg, His**, Pro, Asn, Ser	[Thr, Ala, Gly, Lys, Gln]	Phe, Tyr, Cys, Leu, Ile, Val, Asp (Glu, Met, Trp)
14	**Cys**	(Other amino acids ONLY if C_{38} also changed.)	
15	**Arg, Lys**	[Ala, Ser, Gly, Met, Asn, Gln]	(Cys, Asp, Glu, Phe, His, Ile, Leu, Pro, Thr, Val, Trp, Tyr)
16	**Ala, Gly**	[Ser, Asp, Asn]	(Cys, Glu, Phe, His, Ile, Lys, Leu, Met, Pro, Gln, Arg, Thr, Val, Trp, Tyr)
17	**Ala, Asn**, Ser, Ile	[Gly, Val, Gln, Thr	Cys, Asp, Phe, His, Pro, Arg, Tyr, (Glu, Lys, Met, Trp)
18	**His**, Leu, Gln	[Ala,	Cys, Asp, Glu, Phe, Gly, Ile, Lys, Met, Asn, Pro, Arg, Ser, Thr, Val, Trp, Tyr
19	**Pro, Gln, Leu**	[Asn, Ile]	Ala, Glu, Gly, Met, Arg, Ser, Thr, Val, Trp, (Cys, Asp, Phe, His, Tyr)
20	**Arg**	Leu, Ala, Ser, Lys, Gln, Val	(Cys, Asp, Glu, Phe, Gly, His, Ile, Met, Asn, Pro, Thr, Trp, Tyr)

Table 15: Substitution to confer high affinity for pKA on KuDoms			
Position	Preferred	Allowed	Unlikely to work
21	**Trp, Phe**	[Tyr, His, Ile]	Cys, Leu (Ala, Asp, Glu, Gly, Lys, Met, Asn, Pro, Gln, Arg, Ser, Thr, Val)
31	**Glu**	[Asp, Gln, Asn, Ser, Ala, Val, Leu, Ile, Thr]	(Arg, Lys, Cys, Phe, Gly, His, Met, Pro, Trp, Tyr)
32	**Glu, Gln**	[Asp, Asn, Pro, Thr, Leu, Ser, Ala, Gly, Val]	(Cys, Phe, His, Ile, Lys, Met, Arg, Trp, Tyr)
33	**Phe**	[Tyr]	other 18 excluded.
34	**Ser, Thr, Ile**	[Val, Ala, Asn, Gly, Leu]	Cys, Asp, Glu, Phe, His, Lys, Met, Pro, Gln, Arg, Trp, Tyr
35	**Tyr**	[Trp, Phe]	(other 17)
36	**Gly**	Ser, Ala	(other 17)
37	**Gly**	(Other amino-acid types allowed only if C14-C38 replaced by other types.)	
38	**Cys**	(Other amino acids ONLY if C_{14} also changed.)	
39	**Gly, Glu, Ala**	[Ser, Asp]	Other 15.

Under "Preferred", most highly preferred type are **bold**

Under "Allowed" are types not actually tested, but judged to be acceptable. Types shown in square brackets were allowed and not selected, but are so similar to types that were selected

that the type is unlikely to abolish pKA binding. Such types are not preferred, but pKA-binding proteins could have such types.

Under "Unlikely to work", types shown outside parentheses have been tried and no isolates had that type; types in parentheses have not been tested, but are judged to be unsuitable from consideration of the types actually excluded.

TABLE 21: First Variegation of LACI-K1

```
        a   b   1   2   3   4   5   6   7   8   9  10
        A   E   M   H   S   F   C   A   F   K   A   D
    |gcc|gag|atg|cat|tcc|ttc|tgc|gcc|ttc|aag|gct|gat|
          | NsiI  |
```

```
                                  I|N
                                  C|H
                              F|S F|Y
                              Y|C L|S L|S
                              L|P W|P W|P
                              H|R Q|R Q|R
                              I|T M|T M|T
                              N|V K|V K|V
                              A|D A|E A|E
        L|P
```

```
  D   G  H|R  C   K  A|G  G  D|G  G   R
 11  12  13  14  15  16  17  18  19  20
|gat|ggt|cNt|tgt|aaa|gSt|NNt|NNS|NNg|cgt|
```

```
  F   F   F   N   I   F   T   R   Q   C
 21  22  23  24  25  26  27  28  29  30
|ttc|ttc|ttc|aac|atc|ttc|acg|cgt|cag|tgc|
                      | MluI  |
```

```
            Q|E                 N|H
            M|K                 C|I
            F|S                 F|Y
            Y|C                 L|S
            L|P                 W|P
            H|R                 Q|R
            I|T                 M|T
            N|V                 K|V
            A|D                 A|E
```

```
 E|Q E|Q  F  G|W  Y   G   G   C  G|D  G   N   Q
 31  32  33  34  35  36  37  38  39  40  41  42
|Sag|Saa|ttc|NNS|tac|ggt|ggt|tgt|NNS|ggt|aac|cag|
                                  | BstEII  |
```

Table 21, continued

```
              N   R   F   E   S   L   E   E
             43  44  45  46  47  48  49  50
            |aac|cgg|ttc|gaa|tct|cta|gag|gaa|
                    | BstBI |   | XbaI |
               | AgeI |
```

```
    51  52  53  54  55  56  57  58  59  60
     C   K   K   M   C   T   R   D   G   A
  |tgt|aag|aag|atg|tgc|act|cgt|gac|ggc gcc
                                  | KasI  |
```

The segment from *Nsi*I to *Mlu*I gives 65,536 DNA sequences and 31,200 protein sequences. Second group of variegation gives 21,840 and 32,768 variants. This variegation can go in on a fragment having *Mlu*I and one of *Age*I, *Bst*BI, or *Xba*I ends. Because of the closeness between codon 42 and the 3' restriction site, one will make a self-priming oligonucleotide, fill in, and cut with *Mlu*I and, for example, *Bst*BI. Total variants are 2.726×10^9 and 8.59×10^9.

The DNA sequence has SEQ ID NO. 56.

The amino acid sequence has SEQ ID NO. 57.

TABLE 23: Specificity Results

Protein displayed on M13 gIIIp	Immobilized enzyme tested				
	Plasmin	Thrombin	pKA	Trypsin	Trypsin, 2 washes
LACI-K1	1	1	1	1	1
KkII/3(D)	3.4	1.5	196.	2.	1.4
BPTI	88.	1.1	1.7	0.3	.8

Numbers refer to relative binding of phage display clones compared to the parental phage display.

The KkII/3(D)(Kallikrein) clone retains the parental molecule's affinity for trypsin. KkII/3(D) was selected for binding to pKA.

TABLE 24: Mat α *S. cerevisiae* expression vectors:

Matα1 (Mfα8)

```
            K   R   P   R                                  SEQ ID NO. 58
   5'-...|AAA|AGG|CCT|CGA|G...-3'                          SEQ ID NO. 59
         | StuI  |
               | XhoI |
```

Matα2 (after introduction of a linker into *Stu*I-cut DNA)

```
                                                amino acids: SEQ ID NO. 60
    K   R   E   A   A   E   P   W   G   A   .   .   L   E
5'|AAA|AGG|GAA|GCG|GCC|GAG|CCA|TGG|GGC|GCC|TAA|TAG|CTC|GAG|3'
          | EagI |  | StyI  | KasI  |          | XhoI  |
                                                DNA: SEQ ID NO. 61
```

Matα-LACI-K1, amino acids: SEQ ID NO. 62, DNA: SEQ ID NO. 63

```
              a   b   c   d   1   2   3   4   5   6   7   8
    K   R   E   A   A   E   M   H   S   F   C   A   F   K
5'|AAA|AGG|GAA|GCG|GCC|GAG|atg|cat|tcc|ttc|tgc|gct|ttc|aaa|
          | EagI |  | NsiI  |

     9  10  11  12  13  14  15  16  17  18  19  20
     A   D   D   G   P   C   K   A   I   M   K   R
   |gct|gat|gaC|ggT|ccG|tgt|aaa|gct|atc|atg|aaa|cgt|
          | RsrII |                | BspHI|

    21  22  23  24  25  26  27  28  29  30
     F   F   F   N   I   F   T   R   Q   C
   |ttc|ttc|ttc|aac|att|ttc|acG|cgt|cag|tgc|
                       | MluI  |

    31  32  33  34  35  36  37  38  39  40  41  42
     E   E   F   I   Y   G   G   C   E   G   N   Q
   |gag|gaA|ttC|att|tac|ggt|ggt|tgt|gaa|ggt|aac|cag|
       | EcoRI |                        | BstEII |

            43  44  45  46  47  48  49  50
             N   R   F   E   S   L   E   E
           |aac|cgG|ttc|gaa|tct|ctA|gag|gaa|
             |     | BstBI |  | XbaI |
             | AgeI |

   51  52  53  54  55  56  57  58  59  60
    C   K   K   M   C   T   R   D   G   A
 |tgt|aag|aag|atg|tgc|act|cgt|gac|ggc|gcc|TAA|TAG|CTC|GAG|-3'
                               | KasI  |       | XhoI  |
```

We expect that Matα pre sequence is cleaved before GLU_a-ALA_b-

Table 27: High specificity plasma Kallikrein inhibitors

LACI-K1 (SEQ ID NO. 3)
MHSFCAFKADDGPCKAIMKRFFFNIFTRQCEEFIYGGCEGNQNRFESLEECKKMCTRD
KKII/3#7 (SEQ ID NO. 11)
mhsfcafkaddg**H**ck**ANHQ**rfffniftrqc**EE**f**S**yggc**G**gnqnrfesleeckkmctrd
KKII/3#7-K15A (SEQ ID NO. 64)
mhsfcafkaddghc**A**anhqrfffniftrqceefsyggcggnqnrfesleeckkmctrd
KK2/#13-R15A (SEQ ID NO. 65)
mhsfcafkaDGgRc**A**GAHPrWffniftrqcEEfSyggcGgnqnrfesleeckkmctrd
KK2/#11-R15S (SEQ ID NO. 66)
mhsfcafkaddgpc**S**aahprwffniftrqceefsyggcggnqnrfesleeckkmctrd

Table 49

	Residue Number											
	10	11	12	13	14	15	16	17	18	19	20	21
LACI-K1	D	D	G	P	C	K	A	I	M	K	R	F
Consensus of KKII/3 selectants	d	d	g	H	c	k	A	N	H	Q	r	f
KK Library #2	NK DE	NI AD ST GV	g	FS YC LP HR IT NV AD G	c	KR	AG	NI AD ST GV	QL HP R	QL HP R	r	FW CL
KK2/#13	-	G	-	-	-	-	-	-	-	-	-	-
KK2/#14	-	G	-	-	-	-	-	-	-	-	-	-
KK2/#5	-	-	-	P	-	-	A	-	-	-	-	-
KK2/#11	-	-	-	P	-	-	A	-	-	-	-	-
KK2/#1	-	V	-	-	-	-	-	-	-	-	-	-
KK2/#4	-	V	-	-	-	-	-	-	Q	-	-	F
KK2/#6	-	-	-	S	-	-	A	-	-	L	-	-
KK2/#10	E	G	-	S	-	-	A	-	-	Q	-	-
KK2/#8	-	-	-	P	-	-	-	-	-	L	-	F
KK2/#3	-	-	-	H	-	-	-	-	L	-	-	-
KK2/#9	-	S	-	N	-	-	-	N	L	-	-	F
KK2/#7	-	S	-	-	-	-	-	N	-	Q	-	F
KK2/#12	-	G	-	-	-	-	A	I	Q	-	-	-
Consensus #2	D	D	g	R	c	R	G	A	H	P	r	W

Table 750: DNA that embodies Library KKF.

```
                                                                  N|K
                    M   H   S   F   C   A   F   K   A           D|E
                    1   2   3   4   5   6   7   8   9           10
        5'-cctcct atg cat tcc ttc tgc gcc ttc aag gct           RaS
                  | NsiI  |

              F|S
              Y|C
              L|H
              R|I
      I|V     V|T                 V|T (M)  (K)
      T|A     A|G                 A|S L|P  L|P
      S|G     D|N                 G|D Q|R  R|H
      D|N  G   P   C  K|R A|G  I|N  H   Q   R
      11  12  13  14  15  16  17  18  19  20
      RNt ggt NNt tgt aRa gSt RNt cNS cNS cgt

      W|L
      F|C  F   F   N   I   F   T   R   (SEQ ID NO. 69)
      21  22  23  24  25  26  27  28
      tKS ttc ttc aac atc ttc acg cgt tccctcc-3'  (SEQ ID NO. 67)
             3'-g ttg tag aag tgc gca agggagg-5'  (SEQ ID NO. 68)
                                | MluI  |
```

The *Rsr*II and *Bsp*HI sites found in the parental LACI-K1 display gene (Table 6) are not present in Library KKF.

There are 1,536,000 amino-acid sequences and 4,194,304 DNA sequences. Met_{18} and Lys_{19} are not allowed in Library KKF.

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CLAIMS

1. A kallikrein inhibiting protein which comprises a Kunitz Domain with residues numbered by reference to mature bovine pancreatic trypsin inhibitor, wherein, at each of the residues corresponding to the below identified residues of BPTI, one of the following allowed amino acids is found,

10	Asp, Glu
11	Asp, Gly, Ser, Val
13	His, Pro, Arg, Asn, Ser
15	Arg, Lys
16	Gly, Ala
17	Asn, Ser, Ala, Ile
18	His, Leu, Gln
19	Gln, Leu, Pro
21	Trp, Phe
31	Glu
32	Glu, Gln
34	Ser, Thr, Ile
39	Gly, Glu, Ala.

2. A plasma kallikrein inhibiting protein which comprises a sequence that is substantially homologuous to a reference sequence being selected from the group consisting of

KKII/3 # 1, KKII/3 # 2, KKII/3 # 3, KKII/3 # 4, KKII/3 # 5, KKII/3 # 6, KKII/3 # 7, KKII/3 # 8, KKII/3 # 9, KKII/3 # 10, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, and KK2con1 as defined in Table 2.

3. A method of preventing or treating a disorder attributable to excessive kallikrein activity which comprises administering, to a human or animal subject who would benefit therefrom, a kallikrein-inhibitory amount of the protein or analogue of claim 1 or claim 2.

4. A method of assaying for kallikrein which comprises providing the protein pr analogue of claim 1 or claim 2 in labeled or insolubilized form, and determining whether a complex of said protein and the kallikrein in a sample is formed.

5. A method of purifying kallikrein from a mixure which comprises providing the protein analogue of claim 1 in insolubilized form, and contacting the mixture with said insolubilized protein or analogue so that kallikrein in the mixture is bound.

CLAIMS

1. A kallikrein inhibiting protein which comprises a Kunitz Domain with residues numbered by reference to mature bovine pancreatic trypsin inhibitor, wherein, at each of the residues corresponding to the below identified residues of BPTI, one of the following allowed amino acids is found,

10	Asp, Glu
11	Asp, Gly, Ser, Val
13	His, Pro, Arg, Asn, Ser
15	Arg, Lys
16	Gly, Ala
17	Asn, Ser, Ala, Ile
18	His, Leu, Gln
19	Gln, Leu, Pro
21	Trp, Phe
31	Glu
32	Glu, Gln
34	Ser, Thr, Ile
39	Gly, Glu, Ala.

2. A plasma kallikrein inhibiting protein which comprises a sequence that is substantially homologuous to a reference sequence being selected from the group consisting of KKII/3 # 1, KKII/3 # 2, KKII/3 # 3, KKII/3 # 4, KKII/3 # 5, KKII/3 # 6, KKII/3 # 7, KKII/3 # 8, KKII/3 # 9, KKII/3 # 10, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, and KK2con1 as defined in Table 2.

3. A method of preventing or treating a disorder attributable to excessive kallikrein activity which comprises administering, to a human or animal subject who would benefit therefrom, a kallikrein-inhibitory amount of the protein or analogue of claim 1 or claim 2.

4. A method of assaying for kallikrein which comprises providing the protein pr analogue of claim 1 or claim 2 in labeled or insolubilized form, and determining whether a complex of said protein and the kallikrein in a sample is formed.

Replace by AMT 3/6

5. A method of purifying kallikrein from a mixure which comprises providing the protein analogue of claim 1 in insolubilized form, and contacting the mixture with said insolubilized protein or analogue so that kallikrein in the mixture is bound.

PCT

FOR US 19 APR 1995

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No. PCT/US95/00299

International Filing Date (11.01.95) 11 JAN 1995

Name of receiving Office and "PCT International Application"

PCT INTERNATIONAL APPLICATION FOR US

Applicant's or agent's file reference (if desired) (12 characters maximum) MARKLAND1BPCT

Box No. I TITLE OF INVENTION

KALLIKREIN-INHIBITING "KUNITZ DOMAIN" PROTEINS AND ANALOGUES THEROF

Box No. II APPLICANT

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

Protein Engineering Corporation
765 concord Avenue
Cambridge, Massachusetts 02138
UNITED STATES OF AMERICA

☐ This person is also inventor.

Telephone No. 617-868-0868

Facsimile No. 617-868-0898

Teleprinter No.

State *(i.e. country)* of nationality: UNITED STATES OF AMERICA

State *(i.e. country)* of residence: UNITED STATES OF AMERICA

This person is applicant for the purposes of: ☐ all designated States ☒ all designated States except the United States of America ☐ the United States of America only ☐ the States indicated in the Supplemental Box

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

MARKLAND, William
26 Windsor Road
Milford, Massachusetts 01757
UNITED STATES OF AMERICA

This person is:

☐ applicant only

☒ applicant and inventor

☐ inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: UNITED KINGDOM

State *(i.e. country)* of residence: UNITED STATES OF AMERICA

This person is applicant for the purposes of: ☐ all designated States ☐ all designated States except the United States of America ☒ the United States of America only ☐ the States indicated in the Supplemental Box

☒ Further applicants and/or (further) inventors are indicated on a continuation sheet.

Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE

The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: ☒ agent ☐ common representative

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

COOPER, Iver P.
BROWDY AND NEIMARK
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Washington, D.C. 20004
UNITED STATES OF AMERICA

Telephone No. 202-628-5197

Facsimile No. 202-737-3528

Teleprinter No. 248633

☐ Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.

Sheet No. 2 PCT/US 95/00299

Continuation of Box No. III FURTHER APPLICANTS AND/OR (FURTHER) INVENTORS

If none of the following sub-boxes is used, this sheet is not to be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

LADNER, Robert Charles
3827 Green Valley Road
Ijamsville, Maryland 21754
United States of America

This person is:
- [ ] applicant only
- [XX] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality: United States of America

State *(i.e. country)* of residence: United States of America

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [XX] the United States of America only [ ] the States indicated in the Supplemental Box

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
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- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.)*

This person is:
- [ ] applicant only
- [ ] applicant and inventor
- [ ] inventor only *(If this check-box is marked, do not fill in below.)*

State *(i.e. country)* of nationality:

State *(i.e. country)* of residence:

This person is applicant for the purposes of: [ ] all designated States [ ] all designated States except the United States of America [ ] the United States of America only [ ] the States indicated in the Supplemental Box

---

[ ] **Further applicants and/or (further) inventors are indicated on another continuation sheet.**

 PCT/US 95/00299

Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) *(mark the applicable check-boxes; at least one must be marked)*:

Regional Patent

- [ ] **AP** **ARIPO Patent:** **KE** Kenya, **MW** Malawi, **SD** Sudan, and any other State which is a Contracting State of the Harare Protocol and of the PCT
- [XX] **EP** **European Patent:** **AT** Austria, **BE** Belgium, **CH and LI** Switzerland and Liechtenstein, **DE** Germany, **DK** Denmark, **ES** Spain, **FR** France, **GB** United Kingdom, **GR** Greece, **IE** Ireland, **IT** Italy, **LU** Luxembourg, **MC** Monaco, **NL** Netherlands, **PT** Portugal, **SE** Sweden, and any other State which is a Contracting State of the European Patent Convention and of the PCT
- [ ] **OA** **OAPI Patent:** **BF** Burkina Faso, **BJ** Benin, **CF** Central African Republic, **CG** Congo, **CI** Côte d'Ivoire, **CM** Cameroon, **GA** Gabon, **GN** Guinea, **ML** Mali, **MR** Mauritania, **NE** Niger, **SN** Senegal, **TD** Chad, **TG** Togo, and any other State which is a member State of OAPI and a Contracting State of the PCT *(if other kind of protection or treatment desired, specify on dotted line)* .....

National Patent *(if other kind of protection or treatment desired, specify on dotted line)*:

- [ ] **AM** Armenia
- [ ] **AT** Austria
- [ ] **AU** Australia
- [ ] **BB** Barbados
- [ ] **BG** Bulgaria
- [ ] **BR** Brazil
- [ ] **BY** Belarus
- [XX] **CA** Canada
- [ ] **CH and LI** Switzerland and Liechtenstein
- [ ] **CN** China
- [ ] **CZ** Czech Republic
- [ ] **DE** Germany
- [ ] **DK** Denmark
- [ ] **ES** Spain
- [ ] **FI** Finland
- [ ] **GB** United Kingdom
- [ ] **GE** Georgia
- [ ] **HU** Hungary
- [XX] **JP** Japan
- [ ] **KE** Kenya
- [ ] **KG** Kyrgyzstan
- [ ] **KP** Democratic People's Republic of Korea .....
- [ ] **KR** Republic of Korea
- [ ] **KZ** Kazakhstan
- [ ] **LK** Sri Lanka
- [ ] **LT** Lithuania
- [ ] **LU** Luxembourg
- [ ] **LV** Latvia
- [ ] **MD** Republic of Moldova
- [ ] **MG** Madagascar
- [ ] **MN** Mongolia
- [ ] **MW** Malawi
- [ ] **NL** Netherlands
- [ ] **NO** Norway
- [ ] **NZ** New Zealand
- [ ] **PL** Poland
- [ ] **PT** Portugal
- [ ] **RO** Romania
- [ ] **RU** Russian Federation
- [ ] **SD** Sudan
- [ ] **SE** Sweden
- [ ] **SI** Slovenia
- [ ] **SK** Slovakia
- [ ] **TJ** Tajikistan
- [ ] **TT** Trinidad and Tobago
- [ ] **UA** Ukraine
- [XX] **US** United States of America. Continuation-In-Part
- [ ] **UZ** Uzbekistan
- [ ] **VN** Viet Nam

Check-boxes reserved for designating States (for the purposes of a national patent) which have become party to the PCT after issuance of this sheet:

- [ ] .....
- [ ] .....
- [ ] .....
- [ ] .....
- [ ] .....
- [ ] .....

In addition to the designations made above, the applicant also makes under Rule 4.9(b) **all designations which would be permitted** under the PCT except the designation(s) of _____

The applicant declares that those additional designations are subject to confirmation and that any designation which is not confirmed before the expiration of 15 months from the priority date is to be regarded as withdrawn by the applicant at the expiration of that time limit. *(Confirmation of a designation consists of the filing of a notice specifying that designation and the payment of the designation and confirmation fees. Confirmation must reach the receiving Office within the 15-month time limit.)*

Supplemental Box *If the supplemental Box is not used, this sheet need not be included in the request.*

Use this box in the following cases:

1. If, in any of the Boxes, the space is insufficient to furnish all the information:

in such case, write "Continuation of Box No. ..." [indicate the number of the Box] and furnish the information in the same manner as required according to the captions of the Box in which the space was insufficient;

in particular:

(i) if more than two persons are involved as applicants and/or inventors and no "continuation sheet" is available:

in such case, write "Continuation of Box No. III" and indicate for each additional person the same type of information as required in Box No. III;

(ii) if, in Box No. II or in any of the sub-boxes of Box No. III, the indication "the States indicated in the Supplemental Box" is checked:

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the applicant(s) involved and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, European or OAPI patent) for the purposes of which the named person is applicant;

(iii) if, in Box No. II or in any of the sub-boxes of Box No. III, the inventor or the inventor/applicant is not inventor for the purposes of all designated States or for the purposes of the United States of America:

in such case, write "Continuation of Box No. II" or "Continuation of Box No. III" or "Continuation of Boxes No. II and No. III" (as the case may be), indicate the name of the inventor(s) and, next to (each) such name, the State(s) (and/or, where applicable, ARIPO, European or OAPI patent) for the purposes of which the named person is inventor;

(iv) if, in addition to the agent(s) indicated in Box No. IV, there are further agents:

in such case, write "Continuation of Box No. IV" and indicate for each further agent the same type of information as required in Box No. IV;

(v) if, in Box No. V, the name of any State (or OAPI) is accompanied by the indication "patent of addition," or "certificate of addition," or if, in Box No. V, the name of the United States of America is accompanied by an indication "Continuation" or "Continuation-in-part":

in such case, write "Continuation of Box No. V" and the name of each State involved (or OAPI), and after the name of each such State (or OAPI), the number of the parent title or parent application and the date of grant of the parent title or filing of the parent application;

(vi) if there are more than three earlier applications whose priority is claimed:

in such case, write "Continuation of Box No. VI" and indicate for each additional earlier application the same type of information as required in Box No. VI.

2. If the applicant claims, in respect of any designated Office, the benefits of provisions of the national law concerning non-prejudicial disclosures or exceptions to lack of novelty:

in such case, write "Statement Concerning Non-Prejudicial Disclosures or Exceptions to Lack of Novelty" and furnish that statement below.

Continuation of Box V:

This application is a continuation-in-part of USSN 08/208,264, filed 10 March 1994, now pending, which is a continuation-in-part of USSN 08/179,964, filed 11 January 1994, now abandoned.

(10.03.94) (11.01.94)

PCT/US 95 / 00299

Box No. VI PRIORITY CLAIM Further priority claims are indicated in the Supplemental Box ☐

The priority of the following earlier application(s) is hereby claimed:

Country (in which, or for which, the application was filed)	Filing Date (day/month/year)	Application No.	Office of filing (only for regional or international application)
item (1) US	(11.01.94) 11 January 1994	08/179,964	
item (2) US	(10.03.94) 10 March 1994	08/208,264	
item (3)			

Mark the following check-box if the certified copy of the earlier application is to be issued by the Office which for the purposes of the present international application is the receiving Office (a fee may be required):

☒ The receiving Office is hereby requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) identified above as item(s): 1 and 2

Box No. VII INTERNATIONAL SEARCHING AUTHORITY

Choice of International Searching Authority (ISA) *(If two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):* ISA / EP

Earlier search *Fill in where a search (international, international-type or other) by the International Searching Authority has already been carried out or requested and the Authority is now requested to base the international search, to the extent possible, on the results of that earlier search. Identify such search or request either by reference to the relevant application (or the translation thereof) or by reference to the search request:*

Country (or regional Office): Date *(day/month/year)*: Number:

Box No. VIII CHECK LIST

This international application contains the following number of sheets:

1. request	5	sheets
2. description	43	sheets
3. claims	2	sheets
4. abstract	0	sheets
5. drawings	0	sheets
Total	50	sheets

This international application is accompanied by the item(s) marked below:

1. ☐ separate signed power of attorney
2. ☐ copy of general power of attorney
3. ☐ statement explaining lack of signature
4. ☐ priority document(s) *identified in Box No. VI as item(s):*
5. ☐ fee calculation sheet
6. ☐ separate indications concerning deposited microorganisms
7. ☐ nucleotide and/or amino acid sequence listing (diskette)
8. ☐ other *(specify)*:

Figure No. _____ of the drawings (if any) should accompany the abstract when it is published.

Box No. IX SIGNATURE OF APPLICANT OR AGENT

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

Iver P. Cooper (Agent)

For receiving Office use only (11.01.95)

1. Date of actual receipt of the purported international application: 05 Rec'd PCT/PTO 11 JAN 1995
3. Corrected date of actual receipt due to later but timely received papers or drawings completing the purported international application:
4. Date of timely receipt of the required corrections under PCT Article 11(2):
5. International Searching Authority specified by the applicant: ISA / [illegible]
6. ☒ Transmittal of search copy delayed until search fee is paid

2. Drawings:
☐ received:
☐ not received:

For International Bureau use only

Date of receipt of the record copy by the International Bureau: 06 MARCH 1995 (06.03.95)

See Notes to the request form

INTERNATIONAL SEARCH REPORT

Interna[redacted]al Application No
P[redacted]S 95/00299

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/81 A61K38/57 C12Q1/37 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-93 14122 (NOVONORDISK AS) 22 July 1993 see claims ---	1-5
Y	WO-A-93 14121 (NOVONORDISK AS) 22 July 1993 see claims ---	1-5
Y	WO-A-93 14120 (NOVONORDISK AS) 22 July 1993 see claims --- -/--	1-5

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1995	16.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, vol. 338, 6 April 1989 pages 518-520, T. GIRARD ET AL 'Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor' see the whole document -----	1-5

1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00299

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 3
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 3 is directed to a method treatment of the human bodies the search has been carried out and based on the alleged effect of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

[redacted]mation on patent family members

Interna[redacted]l Application No
[redacted]CT/US 95/00299

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9314122	22-07-93	AU-B-	3346093	03-08-93
		CA-A-	2127246	22-07-93
		CZ-A-	9401644	15-12-94
		EP-A-	0621872	02-11-94
		FI-A-	943234	06-07-94
		JP-T-	7504891	01-06-95
		NO-A-	942549	07-09-94
		ZA-A-	9300096	10-08-93
WO-A-9314121	22-07-93	AU-B-	3345993	03-08-93
		CA-A-	2127249	22-07-93
		CZ-A-	9401649	15-12-94
		EP-A-	0621871	02-11-94
		FI-A-	943233	06-07-94
		NO-A-	942550	07-09-94
		ZA-A-	9300095	20-08-93
WO-A-9314120	22-07-93	AU-B-	3345893	03-08-93
		CA-A-	2127250	22-07-93
		CZ-A-	9401645	15-12-94
		EP-A-	0621870	02-11-94
		FI-A-	943232	06-07-94
		NO-A-	942551	07-09-94
		ZA-A-	9300097	10-08-93

PATENT COOPERATION TREATY

5050

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF RECEIPT OF RECORD COPY

(PCT Rule 24.2(a))

To:

COOPER, Iver, P.
Browdy and Neimark
419 Seventh Street, N.W.
300
Washington, DC 20004
ETATS-UNIS D'AMERIQUE

Date of mailing (day/month/year)	IMPORTANT NOTIFICATION
06 March 1995 (06.03.95)	
Applicant's or agent's file reference	**International application No.**
Markland1Bpc	PCT/US95/00299

The applicant is hereby notified that the International Bureau has received the record copy of the international application as detailed below.

Name(s) of the applicant(s) and State(s) for which they are applicants:

PROTEIN ENGINEERING CORPORATION (for all designated States except US)
MARKLAND, William et al (for US)

International filing date	:	11 January 1995 (11.01.95)
Priority date(s) claimed	:	11 January 1994 (11.01.94) 10 March 1994 (10.03.94)
Date of receipt of the record copy by the International Bureau	:	06 March 1995 (06.03.95)

Designated Offices which will be notified of the receipt of the record copy :

EP :AT,BE,CH,DE,DK,ES,FR,GB,GR,IE,IT,LU,MC,NL,PT,SE
National :CA,JP,US

ATTENTION

The applicant should carefully check the data appearing in this Notification. In case of any discrepancy between these data and the indications in the international application, the applicant should immediately inform the International Bureau.

In addition, the applicant's attention is drawn to the information contained in the Annex, relating to:

[X] time limits for entry into the national phase;

[X] confirmation of precautionary designations;

[X] requirements regarding priority documents.

A copy of this Notification is being sent to the receiving Office and to the International Searching Authority.

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer: J. Leitao
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 730.91.11

Copy for the designated Office (DO/ US

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION IN CASES FOR WHICH NO OTHER FORM IS APPLICABLE

To:

COOPER, Iver, P.
Browdy and Neimark
419 Seventh Street, N.W., #300
Washington, D.C. 20004
ETATS-UNIS D'AMERIQUE

Date of mailing *(day/month/year)* 18 July 1995 (18.07.95)	
Applicant's or agent's file reference MARKLAND1BPC	REPLY DUE see paragraph 1 below
International application No. PCT/US95/00299	International filing date *(day/month/year)* 11 January 1995 (11.01.95)
Applicant PROTEIN ENGINEERING CORPORATION MARKLAND, William, et al.	

1. ☐ REPLY DUE within _____ months/days from the above date of mailing

☐ NO REPLY DUE, however, see below _____

☒ IMPORTANT COMMUNICATION

☐ INFORMATION ONLY

2. COMMUNICATION:

The International Bureau (WO) regrets to inform the applicant that due to a clerical error the above identified international application has not been published promptly after the expiration of 18 months from the priority, as provided in PCT Article 21(2((a).

International publication will take place on **17 August 1995 (17.08.95).**

Meanwhile, the International Bureau (WO) will communicate a copy of the international application to each designated Office, in accordance with PCT Article 20.

A copy of this notification has been sent to the receiving Office (RO/US), the International Searching Authority (ISA/EP) and to all designated Offices concerned.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Leitao
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 730.91.11

Form PCT/IB/345 (July 1992)

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

United States Patent and Trademark Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as elected Office

Date of mailing (day/month/year) 27 September 1995 (27.09.95)	
International application No. PCT/US95/00299	**Applicant's or agent's file reference** Markland1Bpc
International filing date (day/month/year) 11 January 1995 (11.01.95)	**Priority date** (day/month/year) 11 January 1994 (11.01.94)
Applicant MARKLAND, William et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

10 August 1995 (10.08.95)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer

B. Morariu

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as designated Office

Date of mailing:
18 July 1995 (18.07.95)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/US95/00299	

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 730.91.11

08/676,125

PATENT COOPERATION TREATY

RECD 25 APR 1996
WIPO PCT

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference MARKLAND1BPCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US95/00299	International filing date *(day/month/year)* 11 JANUARY 1995	Priority date *(day/month/year)* 11 JANUARY 1994
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PROTEIN ENGINEERING CORPORATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   [✓] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   - I [X] Basis of the report
   - II [ ] Priority
   - III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability
   - IV [ ] Lack of unity of invention
   - V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI [ ] Certain documents cited
   - VII [ ] Certain defects in the international application
   - VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10 AUGUST 1995	01 APRIL 1996
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. DEGEN Telephone No. (703) 308-3672

Form PCT/IPEA/409 (cover sheet) (January 1994)*

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US95/00299

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

☐ the international application as originally filed.

☒ the description, pages (See Attached) , as originally filed.
pages _____ , filed with the demand.
pages _____ , filed with the letter of _____.
pages _____ , filed with the letter of _____.

☒ the claims, Nos. (See Attached) , as originally filed.
Nos. _____ , as amended under Article 19.
Nos. _____ , filed with the demand.
Nos. _____ , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

☒ the drawings, sheets/~~fig~~ (See Attached) , as originally filed.
sheets/~~fig~~ _____ , filed with the demand.
sheets/~~fig~~ _____ , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE.

☒ the claims, Nos. NONE.

☒ the drawings, sheets/~~fig~~ NONE.

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US95/00299

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-7	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-7	NO
Industrial Applicability (IA)	Claims	1-7	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Novo Nordisk 93/14121.

Novo Nordisk '21 discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Novo Nordisk '21 does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Novo Nordisk 93/14122.

Novo Nordisk '22 discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Novo Nordisk '22 does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Girard.
(Continued on Supplemental Sheet.)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US95/00299

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII ~~Sheet 10~~

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): C07K 14/81; A61K 38/57; C12Q 1/37; C12N 9/64 and US Cl.: 530/350, 380, 829, 830; 514/12, 21

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
pages, 1-43, as originally filed.
pages, NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
numbers, NONE, as originally filed.
numbers, NONE, as amended under Article 19.
numbers, NONE, filed with the demand.
and additional amendments:
Claims 1-7, filed with the letter of 05 March 1996.

This report has been drawn on the basis of the drawings,
sheets, NONE, as originally filed.
sheets, NONE, filed with the demand.
and additional amendments:
NONE

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

Girard discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Girard does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Applicants argue that the Novo Nordisk World Patent Applications only teach mutants and procedures that are "obvious to try". However, these arguments are directed towards the WO 93/14120 patent, and both the WO 93/14121 and WO 93/14122 patents teach amino acid substitutions that are either the same as those claimed by Applicants or those that are conservative amino acid substitutions. For example, Applicants claim, at position 31, a Glu residue, which is taught by both the WO 93/14121 and WO 93/14122 patents. See WO 93/14121, page 7, sequences 3-6 and WO 93/14122, page 7, sequence 2. Where Applicants argue that the k_is for the different variants are different, there are no claim limitations directed to the k_is of the proteins.

Claims 1-7 meet the criteria set out in PCT Article 33(4), because these proteins are useful as therapeutics.

CLAIMS

1. A kallikrein inhibiting protein which comprises a non-naturally occurring Kunitz domain, wherein, at each of the residues of said domain corresponding to the below identified residues of BPTI, one of the following allowed amino acids is found:

BPTI residue #	Allowed Amino Acid
10	Asp, Glu, Ala, Gly, Ser, Thr
11	Asp, Gly, Ser, Val, Glu, Leu, Met, Asn, Ile, Ala, Thr
12	Gly, and, if residue 14 or 38 is not Cys, any conservative or semi-conservative substitution for a "normal" conformation Gly as defined in Table 9
13	Arg, His, Pro, Asn, Ser, Thr, Ala, Gly, Lys, Gln
14	Cys, and, if residue 38 is not Cys, any conservative or semi-conservative substitution for Cys
15	Arg, Lys, Ala, Ser, Gly, Met, Asn, Gln
16	Ala, Gly, Ser, Asp, Asn
17	Ala, Asn, Ser, Ile, Gly, Val, Gln, Thr
18	His, Leu, Gln, Ala
19	Pro, Gln, Leu, Asn, Ile
20	Arg, Leu, Ala, Ser, Lys, Gln, Val

21	Trp, Phe, Tyr, His, Ile
31	Glu, Asp, Gln, Asn, Ser, Ala, Val, Leu, Ile, Thr
32	Glu, Gln, Asp, Asn, Pro, Thr, Leu, Ser, Ala, Gly, Val
33	Phe, Tyr
34	Ser, Thr, Ile, Val, Ala, Asn, Gly, Leu
35	Tyr, Trp, Phe
36	Gly, Ser, Ala
37	Gly, and, if residue 14 or 38 is not Cys, any conservative or semi-conservative substitution for a "normal" conformation Gly as defined in Table 9
38	Cys, and, if residue 14 is not Cys, any conservative or semi-conservative substitution for Cys
39	Gly, Glu, Ala, Ser, Asp.

2. A kallikrein inhibiting protein which comprises a non-naturally occurring Kunitz domain, wherein, at each of the residues corresponding to the below identified residues, one of the following allowed amino acids is found:

BPTI residue #	Allowed Amino Acid
10	Asp, Glu, Ala, Gly, Ser, Thr
11	Asp, Gly, Ser, Val, Glu, Leu, Met
12	Gly, and, if residue 14 or 38 is not Cys, or any conservative

	semi-conservative substitution for a "normal" conformation Gly as defined in Table 9
13	Arg, His, Pro, Asn, Ser
14	Cys, and, if residue 38 is not Cys, any conservative or semi-conservative substitution for Cys
15	Arg, Lys
16	Ala, Gly
17	Ala, Asn, Ser, Ile
18	His, Leu, Gln
19	Pro, Gln, Leu
20	Arg, Leu, Ala, Ser, Lys, Gln, Val
21	Trp, Phe
31	Glu
32	Glu, Gln
33	Phe
34	Ser, Thr, Ile
35	Tyr
36	Gly, Ser, Ala
37	Gly, and, if residue 14 or 38 not Cys, any conservative or semi-conservative substitution for a "normal" conformation Gly as defined in Table 9
38	Cys, and, if residue corresponding to position 14 is not Cys, any conservative or semi-conservative substitution for Cys

39	Gly, Glu, Ala.

3. The protein of claim 2 wherein, the Kunitz domain is further characterized as follows:

BPTI Residue No.	Allowed Residue
10	Asp, Glu
11	Asp, Gly, Ser, Val
12	Gly
14	Cys
20	Arg
36	Gly
37	Gly
38	Cys.

4. A plasma kallikrein inhibiting protein which comprises a sequence that is substantially homologuous to a reference sequence selected from the group consisting of

KKII/3 #1, KKII/3 #2, KKII/3 #3, KKII/3 #4, KKII/3 #5, KKII/3 #6, KKII/3 #7, KKII/3 #8, KKII/4 #9, KKII/3 #10, KK2/#11, KK2/#13, KK2/#1, KK2/#2, KK2/#3, KK2/#4, KK2/#6, KK2/#7, KK2/#8, KK2/#9, KK2/#10, KK2/#12, AND KK2con1 as defined in Table 2.

5. A method of preventing or treating a disorder attributable to excessive kallikrein activity which comprises administering, to a human or animal subject who would benefit therefrom, a kallikrein-inhibitory amount of the protein of any of claims 1-4.

6. A method of assaying for kallikrein which comprises providing

the protein of any of claims 1-4 in labeled or insolubilized form, and determining whether a complex of said protein and the kallikrein in a sample is formed.

7. A method of purifying kallikrein from a mixture which comprises providing the protein of any of claims 1-4 in insolubilized form, and contacting the mixture with said insolubilized protein or analogue so that kallikrein in the mixture is bound.

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
Markland1Bpc		
International application No.	International filing date (day/month/year)	(Earliest) Priority Date (day/month/year)
PCT/US95/00299	11/01/95	11/01/94
Applicant		
PROTEIN ENGINEERING CORPORATION et al.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. [X] **Certain claims were found unsearchable** (see Box I).

2. [ ] **Unity of invention is lacking** (see Box II).

3. [X] The international application contains disclosure of a **nucleotide and/or amino acid sequence listing** and the international search was carried out on the basis of the sequence listing

   - [ ] filed with the international application.
   - [X] furnished by the applicant separately from the international application,
     - [ ] but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
   - [ ] Transcribed by this Authority

4. With regard to the **title,**
   - [X] the text is approved as submitted by the applicant.
   - [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**
   - [ ] the text is approved as submitted by the applicant.
   - [X] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is:

   Figure No. _____

   - [ ] as suggested by the applicant.
   - [ ] because the applicant failed to suggest a figure.
   - [ ] because this figure better characterizes the invention.
   - [ ] None of the figures.

INTERNATIONAL SEARCH REPORT

[redacted]national application No.
PCT/US95/00299

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 3
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 3 is directed to a method treatment of the human bodies the search has been carried out and based on the alleged effect of the compound.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

Box III TEXT OF THE ABSTRACT (Continuation of item 5 of the first sheet)

SUMMARY OF THE INVENTION

This invention relates to novel BPTI-homologous Kunitz domains, especially LACI homologues, which inhibit one or more plasma (and/or tissue) kallikreins, and to the therapeutic and diagnostic use of these novel proteins. In particular, this invention relates to Kunitz domains derived from Kunitz domains of human origin and especially to the first Kunitz domain of LACI.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 95/00299

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/81 A61K38/57 C12Q1/37 C12N9/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO-A-93 14122 (NOVONORDISK AS) 22 July 1993 see claims ---	1-5
Y	WO-A-93 14121 (NOVONORDISK AS) 22 July 1993 see claims ---	1-5
Y	WO-A-93 14120 (NOVONORDISK AS) 22 July 1993 see claims ---	1-5
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 July 1995	16.08.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	NATURE, vol. 338, 6 April 1989 pages 518-520, T. GIRARD ET AL 'Functional significance of the Kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor' see the whole document -----	1-5

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 95/00299

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9314122	22-07-93	AU-B-	3346093	03-08-93
		CA-A-	2127246	22-07-93
		CZ-A-	9401644	15-12-94
		EP-A-	0621872	02-11-94
		FI-A-	943234	06-07-94
		JP-T-	7504891	01-06-95
		NO-A-	942549	07-09-94
		ZA-A-	9300096	10-08-93
WO-A-9314121	22-07-93	AU-B-	3345993	03-08-93
		CA-A-	2127249	22-07-93
		CZ-A-	9401649	15-12-94
		EP-A-	0621871	02-11-94
		FI-A-	943233	06-07-94
		NO-A-	942550	07-09-94
		ZA-A-	9300095	20-08-93
WO-A-9314120	22-07-93	AU-B-	3345893	03-08-93
		CA-A-	2127250	22-07-93
		CZ-A-	9401645	15-12-94
		EP-A-	0621870	02-11-94
		FI-A-	943232	06-07-94
		NO-A-	942551	07-09-94
		ZA-A-	9300097	10-08-93

PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

COMMUNICATION OF
INTERNATIONAL APPLICATIONS

(PCT Article 20)

To:

United States Patent and Trademark
Office
(Box PCT)
Washington D.C. 20231
United States of America

in its capacity as designated Office

Date of mailing:
21 September 1995 (21.09.95)

The International Bureau transmits herewith copies of the international applications having the following international application numbers and international publication numbers:

International application no.:	International publication no.:
PCT/US95/00299	WO95/21601

CORRECTED VERSION
VERSION CORRIGEE

The International Bureau of WIPO
34, chemin des Colombettes
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 730.91.11

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To: IVER P. COOPER
BROWDY AND NEIMARK
419 SEVENTH STREET N. W., STE. 300
WASHINGTON, D. C. 20004

PCT

NOTIFICATION OF TRANSMITTAL OF INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Rule 71.1)

Date of Mailing *(day/month/year)*	18 APR 1996
Applicant's or agent's file reference MARKLAND1BPCT	IMPORTANT NOTIFICATION

International application No.	International filing date *(day/month/year)*	Priority Date *(day/month/year)*
PCT/US95/00299	11 JANUARY 1995	11 JANUARY 1994

Applicant
PROTEIN ENGINEERING CORPORATION

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices)(Article 39(1))(see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	NANCY J. DEGEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-3672

Form PCT/IPEA/416 (July 1992)*

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference MARKLAND1BPCT	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/US95/00299	International filing date *(day/month/year)* 11 JANUARY 1995	Priority date *(day/month/year)* 11 JANUARY 1994
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant PROTEIN ENGINEERING CORPORATION		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets.

   [✓] This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

   I [X] Basis of the report

   II [ ] Priority

   III [ ] Non-establishment of report with regard to novelty, inventive step or industrial applicability

   IV [ ] Lack of unity of invention

   V [X] Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

   VI [ ] Certain documents cited

   VII [ ] Certain defects in the international application

   VIII [ ] Certain observations on the international application

Date of submission of the demand	Date of completion of this report
10 AUGUST 1995	01 APRIL 1996
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer NANCY J. DEGEN Telephone No. (703) 308-3672

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US95/00299

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments)*:

☐ the international application as originally filed.

☒ the description, pages (See Attached) , as originally filed.
pages _____ , filed with the demand.
pages _____ , filed with the letter of _____.
pages _____ , filed with the letter of _____.

☒ the claims, Nos. (See Attached) , as originally filed.
Nos. _____ , as amended under Article 19.
Nos. _____ , filed with the demand.
Nos. _____ , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.

☒ the drawings, sheets/~~fig~~ (See Attached) , as originally filed.
sheets/~~fig~~ _____ , filed with the demand.
sheets/~~fig~~ _____ , filed with the letter of _____.
sheets/~~fig~~ _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

☒ the description, pages NONE .

☒ the claims, Nos. NONE .

☒ the drawings, sheets/~~fig~~ NONE .

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the ~~Supplemental Box~~ Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.
PCT/US95/00299

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. STATEMENT

Novelty (N)	Claims	1-7	YES
	Claims	NONE	NO
Inventive Step (IS)	Claims	NONE	YES
	Claims	1-7	NO
Industrial Applicability (IA)	Claims	1-7	YES
	Claims	NONE	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Novo Nordisk 93/14121.

Novo Nordisk '21 discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Novo Nordisk '21 does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Novo Nordisk 93/14122.

Novo Nordisk '22 discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Novo Nordisk '22 does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Claims 1-7 lack an inventive step under PCT Article 33(3) as being obvious over Girard.
(Continued on Supplemental Sheet.)

Supplemental Box
(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

CLASSIFICATION:
The International Patent Classification (IPC) and/or the National classification are as listed below:
IPC(6): C07K 14/81; A61K 38/57; C12Q 1/37; C12N 9/64 and US Cl.: 530/350, 380, 829, 830; 514/12, 21

I. BASIS OF REPORT:

This report has been drawn on the basis of the description,
pages, 1-43, as originally filed.
pages, NONE, filed with the demand.
and additional amendments:
NONE

This report has been drawn on the basis of the claims,
numbers, NONE, as originally filed.
numbers, NONE, as amended under Article 19.
numbers, NONE, filed with the demand.
and additional amendments:
Claims 1-7, filed with the letter of 05 March 1996.

This report has been drawn on the basis of the drawings,
sheets, NONE, as originally filed.
sheets, NONE, filed with the demand.
and additional amendments:
NONE

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

Girard discloses human Kunitz-type protease inhibitor variants with particular sequences. These variants may be used as pharmaceutical compositions to treat various conditions such as pancreatitis, inflammation, thrombocytopenia and many others. The inhibitor variants may also be used as diagnostic agents in screening assays useful in isolating other natural substances for medical purposes. The variants may be isolated by precipitation or chromatographic means. Girard does not teach the exact sequences claimed by Applicants. It would have been obvious to one with ordinary skill in the art at the time Applicants' invention was made to substitute one amino acid for another, particularly in areas of a protein that are not in the active portion of the polypeptide or with an amino acid with similar properties.

Applicants argue that the Novo Nordisk World Patent Applications only teach mutants and procedures that are "obvious to try". However, these arguments are directed towards the WO 93/14120 patent, and both the WO 93/14121 and WO 93/14122 patents teach amino acid substitutions that are either the same as those claimed by Applicants or those that are conservative amino acid substitutions. For example, Applicants claim, at position 31, a Glu residue, which is taught by both the WO 93/14121 and WO 93/14122 patents. See WO 93/14121, page 7, sequences 3-6 and WO 93/14122, page 7, sequence 2. Where Applicants argue that the k_is for the different variants are different, there are no claim limitations directed to the k_is of the proteins.

Claims 1-7 meet the criteria set out in PCT Article 33(4), because these proteins are useful as therapeutics.